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TRAM NGOC QUYNH PHAM

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# PROTEIN KINASE C-ETA (PKC-η) IS REQUIRED FOR THE EXPRESSION OF THE INDUCIBLE NITRIC OXIDE SYNTHASE (NOS II) IN HUMAN MONOCYTIC CELLS: A CORRELATION IN TRANSCRIPTION BETWEEN PKC-η AND NOS II IN INFLAMMATORY ARTHRITIDES

by

**TRAM NGOC QUYNH PHAM**

Thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor in Philosophy

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## ABSTRACT

Nitric oxide (NO), produced by the inducible nitric oxide synthase (NOS II), is important in host defence against invading pathogens including bacteria, fungi and certain viruses. Its induction by LPS and pro-inflammatory cytokines (*e.g.*, TNF-α, IFN-γ, and IL-1β) is well established in murine monocytic cells. However, the same can not be said for human monocytic cells. This work explored the possibility that the failure of human cells to express NOS II following LPS stimulation might be due to a lack of key signalling molecule(s) essential to the pathway.

Since studies with PKC inhibitors have implicated protein kinase C (PKC) in NOS II regulation in murine monocytic cells, a complete investigation comparing PKC isotype expression between human and murine monocytic cells was needed to address whether a lack of one of the isotypes may be responsible for the failure of human monocytic cells to express NOS II. We found that human monocytic cells lacked PKC-η (eta), while the murine counterparts lacked PKC-β1 (beta 1). Subsequently, it was determined that human monocytic cells transfected with PKC-η could induce NOS II expression and produced NO production following LPS stimulation. This provides direct evidence that PKC-η may be involved in NOS II regulation. We have now established expression correlation between PKC-η and NOS II in monocyte-derived macrophages (MDM) from patients with inflammatory diseases (IA) (rheumatoid arthritis [RA] and spondyloarthropathies [SpA]).

Severely- affected IA patients (with > 5 swollen joints) showed highly elevated plasma NO (237.8 ± 34.4 μM, n=10) relative to healthy (131.1 ± 18.9 μM, n=9), osteoarthritis (126.9 ± 40.9 μM, n=13), or mildly affected IA individuals (with < 5 swollen joints) (131.1 ± 18.9 μM, n=10). Analysis of transcriptional expression of NOS II revealed that only in IA patients with elevated plasma NO was NOS II present in MDM, and that PKC-η was always expressed in the NOS II-reactive cases. MDM in individuals with normal circulating NO were negative for both PKC-η and NOS II. This was true in 15 out of 16 (94%) cases tested implicating for the first time a requirement of PKC-η for the development of a NOS II-positive phenotype in human monocytic cells.

In our study, TNF-α did not seem to be involved in NOS II regulation as IA patients (n=7), who responded well clinically to infliximab (anti-TNF-α monoclonal antibody), showed elevated NO and expressed both NOS II and PKC-η in MDM. In contrast, IA patients responding well to anakinra (IL-1 receptor antagonist) had normal plasma NO, did not express NOS II but were positive for PKC-η in MDM. We propose that in severely affected IA, there may be three stages in the development of NOS II positive phenotype in monocytes with IL-1 somehow being involved in the regulation of NOS II expression.

Two synthetic (JPK-101 and JPK-109) and one natural (JPK-113) compounds from Trypterigyum plant, whose extract is used as traditional Chinese medication for the treatment for RA, were investigated for their effects on the

induction of NOS II as well as production of TNF-α, NO, and matrix metalloprotease (MMP). We found that the compounds could inhibit production of NO (via suppression of NOS II) and TNF-α, but not MMP. These findings support their well known, but poorly explained, anti-inflammatory effects.

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plasmids needed for the transfection experiments and Dr. Felley-Bosco (Switzerland) for the pBABE plasmid used in the preparation of the NOS II probe.

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| | |
|---|---|
| APC | Antigen presenting cells |
| AS | Ankylosing spondylitis |
| BPB | Bromophenol blue |
| CBB | Coomassie brilliant blue |
| COX | Cyclooxygenase |
| DAG | Diacylglyceride |
| DAN | 2,3-diaminonaphthalene |
| DMARD | Disease-modifying anti-rheumatic drugs |
| EDTA | Ethylene-diamine-tetraacetic-aci |
| EGTA | Ethylene glycol-bis-(β-aminoethyl ether)-N, N, N', N',-tetra acetic acid |
| ESR | Erythrocyte sedimentation rate |
| EtBr | Ethidium bromide |
| FBS | Fetal bovine serum |
| HEPES | N-2-hydroxylpiperazine-N-2-ethane sulphonic acid |
| IA | Inflammatory arthritis |
| $IC_{50}$ | Inhibitory concentration |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |
| IL-1Ra | Interleukin-1 receptor antagonist |
| LBP | Lipopolysaccharide-binding protein |
| LPS | Lipopolysaccharide |
| MDM | Monocyte-derived macrophage |
| 2-ME | 2- beta mercaptoethanol |
| MMP | Matrix metalloproteases |
| Mo | Monocyte |
| Mϕ | Macrophage |
| MTX | Methotrexate |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NOS II | Inducible nitric oxide synthase |
| OA | Osteoarthritis |
| PBS | Phosphate buffer saline |
| PAMP | Pathogen-associated molecular patterns |
| PFA | Paraformaldehyde |
| PGE2 | Prostaglandin E2 |
| PKC-η | Protein kinase C-eta |
| PMA | Phorbol myristate acetate |
| PMSF | Phenylmethyl-sulfonyl fluoride |

| | |
|---|---|
| PPR | Pathogen-recognition molecules/receptors |
| PS | Phosphoserine |
| PsA | Psoriatic arthritis |
| RA | Rheumatoid arthritis |
| RF | Rheumatoid factor |
| RNI | Reactive nitrogen intermediates |
| ROI | Reactive oxygen intermediates |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| SF | Serum free |
| SpA | Spondyloarthropathy |
| $TC_{50}$ | Toxicity concentration |
| TE | Tris-EDTA |
| TI | Therapeutic index |
| TLR-4 | Toll-like receptor 4 |
| TNF-α | Tumor necrosis factor-α |
| TwHf | Trypterygium *wilfordii Hook* f |

# CHAPTER ONE

# INTRODUCTION

## 1.1 THE MACROPHAGE

### 1.1.1 Development

Monocytes (Mo) and macrophages (Mϕ) are mononuclear cells which perform diverse functions ranging from accessory cell function to microbe, and even tumor cell, killing. Three times bigger than lymphocytes, Mo and Mϕ have a single large kidney-shaped non-segmented nucleus and a well-defined cytoplasm. On average, peripheral blood monocytes constitute 1-5% of the total leukocyte or white blood cell, population.

Ever since the first designation of Mϕ introduced in1892, intense discussion of the origin, development and differentiation of Mϕ has continued. Since then, there have been two major theories proposed for the origin and the developmental process of the Mϕ. Aschoff (1924) put forward the concept that Mϕ were a part of the reticulo-endothelial system together with the reticulum cells and reticuloendothelia. In contrast, Langevoort *et al.* (1970) presented the theory of the "mononuclear phagocyte system" (MPS) which asserted that all Mϕ, including those produced during inflammation (exudate Mϕ) and those residing in tissues during normal conditions (resident Mϕ), are derived from the same precursor cells in the bone marrow. However, there is evidence in the literature (Miyamura *et al.*, 1998) suggesting that exudate and resident Mϕ are indeed two distinct populations, thus contradicting the MPS notion that exudate Mϕ exemplify a transitional or

intermediate form of resident Mϕ. Despite the conflicting data, the core of the MPS theory has gained a lot of support over the years. The current consensus is that a Mo does undergo sequential developmental changes after beginning its life as a hematopoietic stem cell (HSC) in the bone marrow. In the presence of various growth factors including interleukin-3 (IL-3), interleukin-6 (IL-6) and granulocyte macrophage-colony stimulating factor (GM-CSF), the HSC differentiates into a granulocyte-macrophage-colony forming cell (GM-CFC). In an environment rich in macrophage-colony stimulating factor (M-CSF), the latter then progresses into a macrophage colony-forming cell which sequentially develops into a monoblast, a pro-monocyte and then finally a Mo. The Mo then leaves the bone marrow and enters the circulation. After approximately two days, it leaves the circulation, takes up residence in various tissues and becomes a Mϕ. In addition, the bone marrow can and does release immature myeloid GM-CFC into peripheral blood. These cells then migrate to tissues where, in the presence of *in situ* GM-CSF and M-CSF, they differentiate into long-lived resident Mϕ or related cells (*e.g.* Langerhans in the skin and Kupffer cells in the liver). In response to inflammatory stimuli, Mo migrate to the affected tissue and differentiate into exudate Mϕ (Beelen *et al.*, 1978). Resident Mϕ differ from exudate Mϕ in that the former are long-lived in tissue and capable of self-renewal (Takahashi *et al.*, 1996). As their production is only induced as a result of an insult, it is prudent that exudate Mϕ be short-lived and incapable of proliferation.

### 1.1.2 Role of mononuclear phagocytes in immunity

Mϕ are extraordinarily versatile cells. These "cells for all seasons" are found in most tissues in the body participating in a wide range of biological processes including development, bone remodelling, and wound healing (Morrissette *et al.*, 1999). However, it is as sentinels of the immune system that Mϕ exploit their full functional repertoire. When microorganisms such as bacteria invade the tissue, Mϕ are chemotactically attracted to the site of insult and begin to phagocytose. Activated Mϕ are professional phagocytes in that they detect, ingest, and destroy infectious agents. This makes Mϕ essential components of innate immunity. Phagocytosed bacteria are killed, and then degraded by a number of mechanisms, all of which are part of the innate response. Moreover, activated Mϕ also promote the development of an acute inflammatory response through secretion of soluble inflammatory mediators including platelet activating factor (PAF), prostaglandin (PG), leukotrienes (LT), reactive oxygen intermediates (ROI) *(e.g.* hydrogen peroxide and superoxide), reactive nitrogen intermediates (RNI) (*e.g.* nitric oxide [NO]) as well as cytokines including IL-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor (TNF-α). Platelet activating factor is important in the activation of platelets, chemotaxis of eosinophils, as well as activation and degranulation of neutrophils and eosinophils. Prostaglandin increases vascular permeability and

dilation resulting in tissue edema and leukocyte extravasation. Break-down of arachidonic acid via the lipoxygenase pathway in Mo, Mϕ and mast cells gives rise to LT. This lipid inflammatory mediator induces smooth muscle contraction and attracts neutrophils. In addition, activation of two oxidative pathways, NADPH oxidase and inducible nitric oxide synthase (NOS II), also known as the respiratory burst, produces toxic ROI and NO, respectively, exerting injury to the target in a number of different ways (Chapter 3). ROI and RNI account for one of the most powerful weapons of the Mϕ against microbial organisms. Mice with NOS II knock-out are more susceptible to infection with intracellular bacteria than wild type mice (MacMicking *et al.*, 1997). Also, studies with Mϕ deficient in these two enzymes reveal their role in helping to orchestrate the profound transcriptional remodelling that underlies macrophage activation (Ehrt *et al.*, 2001). The three cytokines IL-1, IL-6, and TNF-α produced by activated tissue Mϕ induce many of the local and systemic changes. Included in this list are an increase in vascular permeability, enhanced expression of cell adhesion molecules, induction of chemokines (*e.g.* interleukin-8 [IL-8]), and upregulation of their own production as well as that of others (*e.g.* interleukin 12 [IL-12]) (Picker *et al.*, 1999; Akira, 2000). These cytokines contribute, in many ways, in bringing other immune cells to the site of infection/injury, where they participate in clearance of the invading organism and healing of the tissue. If the invasion by the microorganism persists, the innate inflammatory response will be supplemented by the various elements of acquired

immunity, in which Mϕ play an important role in the development of both humoral and cell-mediated immune response by helping antigen processing and presentation. Activated Mϕ can also further cytokine secretion. For example, IL-1 and IL-8 are involved in the activation of the endothelium, upregulation of cell adhesion molecules and promotion of leukocyte extravasation. IL-6 induces B cell terminal differentiation into IgG-secreting plasma cells. IL-12 helps activate natural killer (NK) cells and promotes differentiation of the Th1 subset. IL-1, -6 and, TNF-α are all involved in the production of acute phase proteins and inducing fever (Uthaisangsook *et al.*, 2002). Although not as powerful an antigen presenting cell (APC) as dendritic cells (DC), activated Mϕ can also present antigen-derived peptides in the context of major histocompatibility class (MHC) II molecules to $CD4^+$ T cells, thus initiating a T cell response. Activated T cells, in turn, secrete cytokines and help activate other immune cells. The overall result is that the host is able to mount a specific response against the invading antigen. Therefore, with all of these capabilities, Mϕ play an important role in bridging the two arms of innate and adaptive immunity.

In adaptive immunity, activated Mϕ, along with DC and B cells, are professional APC which present exogenous foreign peptides to T cells in the context of self MHC molecules, thus initiating T cell responses. The peptides may be generated after the foreign antigen is phagocytosed and degraded by proteases produced in the activated Mϕ.

### 1.1.3 Activation

Mϕ are remarkably versatile cells in that they are responsive to a diverse range of stimuli. Thus, Mϕ can be activated in a number of different ways. First, pathogen- recognition molecules/receptors (PRR) expressed on the surface of the Mϕ can bind to pathogen-associated molecular patterns (PAMPs) on micro-organisms. These PAMPs including lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), lipopeptides, and CpG DNA. The recent discovery of the Toll-like receptor (TLR) family in mammalians (reviewed by Underhill and Ozinsky, 2002) has greatly enhanced our understanding of PAMP recognition by the PRR. Each member of the TLR family (ten have so far been characterized) has been shown to recognize specific components of microbes. For example, TLR-4 is thought to be involved in stabilizing the LPS-CD14 interaction as well as signal transduction mediated by LPS (Arbour *et al.*, 2000), whileTLR-2 has been reported to signal the presence of bacterial lipoproteins (Aliprantis *et al.*, 1999) and lipoteichoic acids (Schwandner *et al.*, 1999). Bacterial CpG DNA, once bound to its ligand TLR-9, becomes a potent inducer of Th1 response and helps induce maturation of DC (Takeda and Akira, 2002). Macrophage activation leads to induction of various stimulatory products including NO (Chapter 3), TNF-α, IL-1 and matrix-degrading enzymes like matrix-metalloproteases (MMP) (Chapter 4). Second, TNF-α and IL-1 once produced by activated Mϕ can bind to their receptors on the Mϕ, thus initiating autocrine stimulatory loops. In addition to being activated

by microbial products and autocrine regulators, as described above, Mϕ can also be activated by products of T lymphocytes, endocytic stimuli, and many physiological hormones. The most studied of the T cell products is inteferon-gamma (IFN-γ). This cytokine helps activate Mϕ such that the cells acquire increased killing potential against intracellular bacteria and tumor cells (Nathan *et al.*, 1984). IFN-γ also helps upregulate the expression of MHC class II molecules on Mϕ allowing them to become more effective APC.

### 1.1.4 Regulation of macrophage function

As important as Mϕ might be in immunity, their activation must be tightly regulated to ensure integrity of the host. In most cases, an excessive immune response is often accompanied by activation of negative feedback mechanisms. In so doing, the host is spared from an overproduction of pro-inflammatory cytokines and other products of activated Mϕ with tissue-damaging potential. However, when and if these controls go awry, Mϕ can become the etiological agents of a number of inflammatory disease processes.

Two best characterized cytokines known to be involved in modulating macrophage activation and functions are tranforming growth factor-β (TGF-β) and interleukin-10 (IL-10) (Assoian *et al.*, 1987). Although TGF-β is classically known as a potent "macrophage-deactivating agent", there is increasing evidence in the literature suggesting that TGF-β can perform dual functions as an activator and

suppressor (Tsunawaki *et al.*, 1988). TGF-β1 is the most abundant form in tissues, and the predominant isoform present at sites of inflammation/tissue injury. It is the major TGF-β isoform secreted by circulating Mo and tissue Mϕ. At the sites of tissue insult, the initial action of platelet degranulation releases TGF-β1, which at femtomolar concentrations, can induce the most potent chemoattractant response in recruiting monocytes to the sites of inflammation or tissue inury (Pierce *et al.*, 1989). Also, femtomolar concentrations of TGF-β1 trigger activation of resting Mo, and subsequent release of cytokines by the activated Mo. TGF-β also mediates an upregulation of Fc gamma receptor III (CD16) on the surface of peripheral blood Mo and mature Mϕ, thus enhancing phagocytosis and removal of cellular debris (Welch *et al.*, 1990). As the inflammatory process progresses, activation and differentiation of Mo (into mature Mϕ) leads to a marked down-regulation of the high affinity type I/II TGF-β receptors on activated Mo at sites of inflammation, thus decreasing sensitivity to TGF-β1 (Brandes *et al.*, 1991). In addition to the receptor modulation mechanism, TGF-β may exert its effects as an anti-inflammatory mediator by inhibiting the release of a number of cytokines/chemokines from activated Mo and Mϕ which are known to promote development of the inflammatory process (*e.g.* monocyte chemotactic protein-1 [MCP-1], monocyte inactivating protein-alpha [MIP-1α], IL-8, GM-CSF, and TNF-α). Alternatively, TGF-β can also influence the expression of (1) receptor or receptor antagonists of pro-inflammatory cytokines (*e.g.* IL-1) (Turner *et al.*, 1991), or (2) anti-inflammatory cytokines (*e.g.* IL-10)

(Ashcroft *et al.*, 1999). These anti-inflammatory effects of TGF-β reflect the importance of the cytokine in the resolution phase of the inflammatory response. Another cytokine that is often associated with anti-inflammation and immune suppression is IL-10. The mode of action for this cytokine is similar to that of TGF-β. IL-10 may attenuate the inflammation process by reducing cytokine production in activated Mϕ through upregulation of suppressor of cytokine signalling-3 (SOCS-3) and of interleukin-1 receptor antagonist (IL-1Ra) (Lang *et al.*, 2002). When produced by Mϕ, IL-10 can bind to its receptor on the Mϕ, thus effectively forming part of an auto-inhibitory autocrine loop, which controls the production of cytokines such as TNF-α (Donnelly *et al.*,1995). In addition, stress hormone glucocorticoids, can also help modulate the inflammatory response by inhibiting the production of pro-inflammatory cytokines by Mϕ (Goldstein *et al.*, 1992).

Recently, Wang and colleagues (2002) provided novel findings for possible regulation by cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) of the immune processes associated with Mo or modulation of Mo/Mϕ functions. The surface marker, which directly competes with CD28 for CD80 and CD86 binding, plays a role in initiating the formation of inhibitory signalling complexes that eventually lead to quiescence or anergy (Sansom *et al.*, 2000). Functional CTLA-4 was found to be constitutively expressed in monocytic cells, and its expression could be up-regulated by PMA and IFN-γ (Wang *et al.*, 2002). In addition to the membrane-bound form, soluble CTLA-4 is also expressed by activated Mo. These

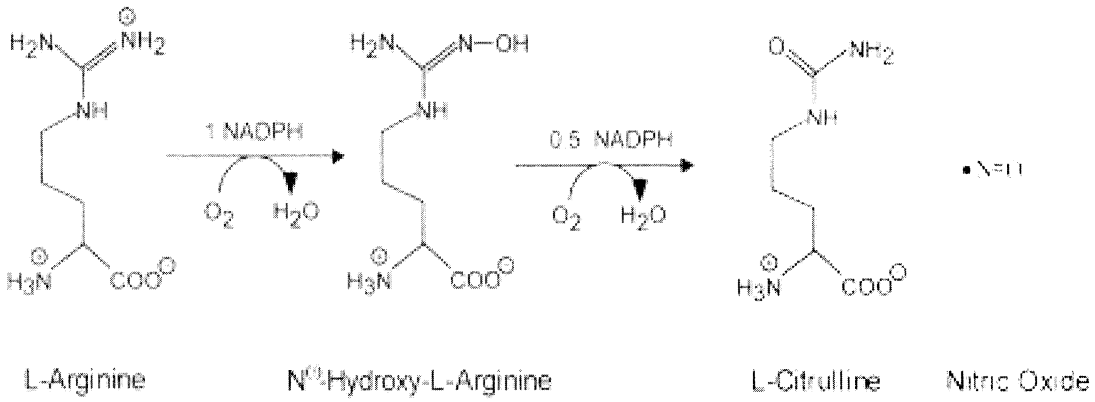
investigators have proposed that ligation of CTLA-4 leads to: (1) inhibition of the activation of a number of transcription factors including NF-κB, and AP-1 in the activated Mo and (2) downregulation of CD86, CD54, HLA -DR and HLA-DQ, which are all crucial to the development of immune responses. Taken together, the balance between anti-inflammatory and inflammatory mediators is absolutely essential to maintaining a healthy host. In fact, this balance is skewed critically, one way or another, in a number of infectious, inflammatory, and allergic diseases. Thus, better understanding of how biochemical processes involved in the production of these molecules may provide critical insights into mechanisms underlying a variety of common human diseases including rheumatoid arthritis (RA). Only then will there be an opportunity for therapeutic intervention for these diseases.

## 1.2 NITRIC OXIDE AND NITRIC OXIDE SYNTHASES

### 1.2.1 Nitric oxide (NO)

NO is generated by a five-electron oxidation of one of the terminal guanidino nitrogen atoms of L-arginine (Fig. 1.1) and the reaction is catalyzed by members of the nitric oxide synthase family. The enzymatic combination of a single nitrogen atom and a single oxygen atom forms NO, the smallest synthetic molecule (30 Dalton) of mammalian cells. To understand why NO was singled out by nature to

**Figure 1.1: Nitric oxide as catalyzed by nitric oxide synthases**




Taken from Marletta (1994) Cell:**78**,927-930

perform diverse biological functions, it is necessary to know the physical properties of this molecule. It has an unpaired electron, which makes the free radical extremely reactive in that it constantly looks for a species that it can either accept an unpaired electron or share this odd one.

### 1.2.2 Nitric oxide synthases (NOS)

To date, there are three members constituting the nitric oxide synthase family (L-arginine, NADPH: oxidoreductases; EC 1.14.13.39). Two of the three NOS members are constitutively expressed: neuronal (nNOS, ncNOS and NOSI) and endothelial (eNOS, ecNOS and NOSIII). The third member of the family is the inducible NOS (iNOS, NOSII) whose expression typically requires cell activation with, among other stimuli, bacterial product lipopolysaccharide (LPS) (Table 1.1). The three NOS isoforms have been isolated, cloned, characterized and they differ in several important aspects including expression characteristics, tissue localization, NO output levels, and regulation of activity. All NOS proteins are protoporphyrin IX heme enzymes which require three co-substrates (L-arginine, NADPH and $O_2$) and five co-factors including FAD, FMN, calmodulin, tetrahydrobiopterin [$BH_4$], and heme.

The three NOS isoenzymes are encoded by different genes. The amino acid sequences deduced from the respective cDNA show that the three proteins share less than 50% sequence homology. However, across species, there is a

**Table 1.1: Nitric oxide synthase isoforms: designation and characteristics**

| | nNOS (NOS I) | iNOS (NOS II) | eNOS (NOSIII) |
|---|---|---|---|
| Human chromosome location | 12q24.2 | 17cen-q12 | 7q35-36 |
| Size of the gene | > 200 kb | 37 kb | 21 kb |
| Number of exons | 29 | 26 | 26 |
| Size of mRNA | 8.5 - 9.5 kb | 4.2 - 4.5 kb | 4.3 - 4.8 |
| Number of amino acid / Protein size (kDa) | 1433 / 161 | 1203 / 131 | 1153 / 133 |
| Subcellular localization | Cytosol / membrane | Cytosol | Membrane |
| Expression of RNA and protein | Constitutive | Inducible | Constitutive |

higher degree of similarity within each type of NOS suggesting a significant phylogenetic conservation. For example, amino acid sequences for NOS I and NOS III, are more than 90% homologous in human, bovine, and rat. Similarly, more than 80% of the NOS II sequence is shared by humans and rodents.

### 1.2.2.1 Neuronal NOS (nNOS, ncNOS, NOS I)

#### 1.2.2.1.1 Expression

NOS I was first named as the neuronal NOS after its discovery in the brain cells of various species (Bredt and Snyder, 1990). Since then, it has been identified in a number of cell types including skeletal muscle cells, pancreatic islet cells, and kidney macula densa cells (Bredt and Snyder, 1994). In the brain, its activity is associated with synaptic elasticity, but in the peripheral nervous system, NOS I is primarily connected to smooth muscle relaxation (Kerwin *et al.*, 1995).

Assigned to the 12q24.2 region of chromosome 12, the human NOS I is by far the largest of the three NOS enzymes with its gene spanning over 150 kb of DNA (Marsden *et al.*, 1993). The mRNA is encoded by 29 exons with the translation initiation and termination sites in exons 2 and 29 respectively, leaving the untranslated region in exon 1. The human NOS I cDNA has an open reading frame of 4299 nucleotides encoding for a protein of 1433 amino acids with a predicted molecular weight of 161 kDa. It is known that NOS I is only capable of producing picomolar quantities of NO.

### 1.2.2.1.2 Regulation of activity and NOS I enzyme expression

The activity of NOS I is primarily regulated by calmodulin in a calcium-dependent manner. The enzyme is generally activated by stimuli which cause a transient increase in intracellular $Ca^{2+}$ levels. With regards to the regulation of NOS I expression, little information is available to this end. However, the presence of the binding sites in the 5'-flanking regions of exon 1 for a number of cis-acting transcription factors including AP-2 (activator protein-2), TEF-1/MCBF (transcription enhance factor-1/M-CAT binding factor), CREB/ATF (cAMP response element binding protein/activating transciption factor), c-fos, NF-1, and NFkB-like sequences suggests that NOS I is likely to be transcriptionally regulated (Hall *et al.*, 1994). There is also evidence supporting a role of estrogen in upregulating NOS I mRNA and activity in various tissues (Weiner *et al.*, 1994). In addition, chronic salt loading of rats also resulted in an increase in mRNA accumulation and protein (Zhang *et al.*, 1994). Functional studies of the human NOS I promoter with reporter gene constructs demonstrate that phorbol esters can increase transcription by two-fold in HeLa cells (Young *et al.*, 1995). These findings, thus, show that NOS I expression can be up-regulated in certain instances. However, the molecular mechanisms behind the phenomenon have yet to be elucidated. Post-transcriptional control of the NOS I gene also exists in the form of alternative mRNA splicing. For instance, 5% of neuronal NOS I transcripts lacked two exons encoding 105 amino acids from position 504 to 608 in the NOS I protein (Ogura *et al.*, 1993).

Similar structural diversity due to mRNA alternative splicing was also found in human cerebellum and differentiated skeletal muscle, where the NOS I protein is a distinct entity from that of the putative neuronal NOS. Together, the variants of NOS I support a notion that alternative mRNA splicing functions as a mechanism for tissue-specific regulation of NOS I.

Mice generated by homozygous deletion of exon 2 exhibited inappropriate sexual and aggressive male behaviour (Nelson *et al.*, 1995) and hypertrophic pyloric stenosis (Vanderwinden *et al.*, 1992). It should be noted that in these mice, the deletion did not lead to a complete absence of neuronal NOS. Rather, due to the alternative mRNA splicing, the lack of exon 2, gives rise to other variants of NOS that may likely explain the lack of histopathological abnormalities in the brain in these animals.

### 1.2.2.2 Inducible NOS (iNOS, NOS II)

#### 1.2.2.2.1 Expression

The human NOS II gene is assigned to the 17q11.2-q12 region of chromosome 17. It consists of 26 exons and 25 introns spanning about 37 kb of human genomic DNA. The translation starts at exon 2 and terminates at exon 26, leaving a 5'-untranslated region of about 22 nucleotides. The full length open reading frame is 3459 base pairs encoding a protein of about 1153 amino acids. NOS II was named

"iNOS" (Xie *et al.*, 1992) to depict its independence of intracellular $Ca^{2+}$ fluxes. Originally identified in macrophages, NOS II, which is localized in the cell cytosol, has since been shown to be expressed in various cell types including hepatocytes (Shiratori *et al.*, 1998), chondrocytes (Grabowski *et al.*, 1996), β-islet cells (Suarez-Pinzon *et al.*, 2001), mesangial cells (Wang and Marsden, 1995), murine mature bone marrow-derived DC (Lu *et al.*, 1996), and murine splenic natural killer cells (Diefenbach *et al.*, 1999). Unlike neuronal and endothelial NOS where the expression is constitutive, that of the NOS II has to be induced. LPS and pro-inflammatory cytokines TNF-α, IL-1 and IFN-γ were among the first immunologic and inflammatory stimuli found to be successful in inducing NOS II. Interleukin-2 (IL-12) IL-18, and IFN-α have all been described as stimulators of NOS II expression (Kroncke *et al.*, 1995). However, the type of cytokine and/ or their combination which induce superior NOS II expression varies greatly among species and between cell types within the same species. In addition to LPS and cytokines, other agents were also identified as inducers of NOS II. Included in this list are: cAMP-elevating agents, such as forskolin or dibutyryl cAMP (Gilbert and Herschman, 1993a; Hortelano *et al.*, 1993), platelet-derived growth factor, and fibroblast growth factor (Gilbert and Herschman, 1993 b). Changes associated with certain disease processes such as RA, diabetes, and AIDS-associated dementia may also lead to the induction of NOS II (St. Clair *et al.*, 1996; Adamson *et al.*, 1999; Suarez-Pinzon *et al.*, 2001). On the other hand, there are a large number of

compounds or molecules thought to be able to prevent the expression of NOS II. For instance, IL-4, IL-8, IL-10, MCP-1, and TGF-β are all thought to be NOS II inhibitors in Mϕ (Bogdan *et al.*, 1994; McCall *et al.*, 1992; Cunha *et al.*, 1992; Rojas *et al.*, 1993; Ding *et al.*, 1990). Glucocorticoids, such as dexamethasone, are effective in inhibiting NOS II induction in a number of cell types including endothelial cells, Mϕ, fibroblasts, and smooth muscle cells (Di Rosa *et al.*, 1990; Gilbert and Herschman *et al.*, 1993 b; Kanno *et al.*, 1993).

### 1.2.2.2.2 Regulation of activity and NOS II enzyme expression

Unlike nNOS and eNOS, NOS II does not depend on $Ca^{2+}$ influx for activation. The reason for the difference is because binding affinity of calmodulin and NOS II is so great that calmodulin can tightly bind to the enzyme even at very low intracellular calcium levels. Given that all three NOS isoforms need the same co-factors, including NADPH, FAD and (6R)-5,6,7,8-tetrahydrobiopterin ($BH_4$), the difference in the activation signal may explain the high and continuous levels of NO produced by NOS II (Iyenger *et al.*, 1987). Primer extension analyses with the RNA of human hepatocytes activated with LPS and IFN-γ mapped the transcription initiation site to 30 base pairs downstream of the TATA box. Functional studies of the cloned murine NOS II promoter have identified a number of sequences/binding motifs that are likely to be involved in the regulation of NOS II expression. For example, there are several consensus sequences for the binding of transcription

factors including IFN-γ-responsive element (IRE), IFN-α-stimulated responsive element, activating protein (AP)-1 site, and a palindromic TNF-α-responsive element like site. Binding sites/motifs for NF-κB and nuclear factor-IL6 (NF-IL6) are also present in multiple copies. Analyses of reporter gene constructs of mutants of the NOS II 5'-flanking region have revealed two functionally important sequences of the 5'- regulatory region. Containing the putative binding sites for NF-IL6 and NF-κB, the first region (positions 49 to 209) is thought to regulate LPS-induced expression of NOS II, as its removal by deletion leads to absence of NOS II induction in LPS-stimulated cells (Lowenstein *et al.*, 1993). The NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) prevented the activation of proteins binding to the NF-κB binding site and the production of NO in LPS-treated murine Mϕ, suggesting that the nuclear factor was essential to NOS II transcription in murine (Xie *et al.*, 1994). The second region spanning residues 913 to 1029 contains a cluster of 4 enhancer motifs known to bind to IFN-γ responsive transcription factors, including an IFN-γ regulatory factor binding site (IRF-E). Deletion of residues within the IRF-E was shown to abolish upregulation of NOS II transcription, as a result of IFN-γ stimulation. Findings such as these support a notion that this second region of the 5'-regulatory sequence might be responsible for the synergistic effect of NOS II induction by IFN-γ and LPS co-stimulation.

As mentioned earlier, a number of cytokines (especially Th2 type cytokines) and other compounds have been identified as inhibitors of NOS II induction. We are

beginning to understand the mechanism whereby these molecules exert their inhibitory function. The evidence shows that TGF-β may: (1) reduce NOS II mRNA levels via decreasing its stability without affecting the transcription; (2) attenuate translation efficiency, and (3) accelerate protein degradation (Vodovotz *et al.*, 1993). On the other hand, interleukin- 4 (IL-4), a Th2 type cytokine, does not appear to negatively affect NOS II stability. Rather, it has been shown to reduce both mRNA expression and protein display 24 to 72 h post stimulation.

NO production can also be regulated by interfering with availability of the main substrate L-arginine. The high output production of NO by NOS II requires extracellular arginine in addition to intracellular arginine. Extracellular arginine is transported inside the cell by a group of carriers known as cationic amino acid transporters (CAT), which themselves are up-regulated after LPS stimulation. Studies with Mϕ from $CAT2^{-/-}$ mice showed more than 90% suppression of arginine uptake and as a result, NO production after LPS or IFN-γ stimulations (Nicholson *et al.*, 2001). Both IL-4, interleukin-13 (IL-13), dexamethasone, and cyclic adenosine mono-phosphate (cAMP) have been found to strongly increase expression of arginase I and arginase II (Munder *et al.*, 1999; Rutschman *et al.*, 2001; Gotoh and Mori, 1999). The up-regulation of arginase prior to NOS II induction by cytokines or LPS prevents NO production by substrate depletion (Munder *et* al., 1999; Rutschman *et al.*, 2001).

Adding to the complexity of the mechanism regulating NOS II expression is the

fact that some molecules may stimulate NOS II induction in one cell type and inhibit in another. For example, as previously discussed TGF-β has been documented to suppress NOS II expression in mouse Mϕ (Ding *et al.*, 1990), but stimulate induction in 3T3 fibroblasts (Gilbert and Herschman, 1993 a,b). Similarly, cAMP and cAMP-elevating agents have been reported to mediate an upregulation of NOS II expression in rat Kupffer cells (Gaillard, 1992) and smooth muscle cells (Koide *et al.*, 1993), but prevent NOS II induction in rat astrocytes (Feinstein *et al.*, 1993). Findings such as these show that signal transduction pathways leading to NOS II induction appear to differ markedly among different cell types of the same species or even within the same cell type of different species.

### 1.2.2.3 Endothelial NOS (eNOS, ecNOS, NOS III)

#### 1.2.2.3.1 Expression

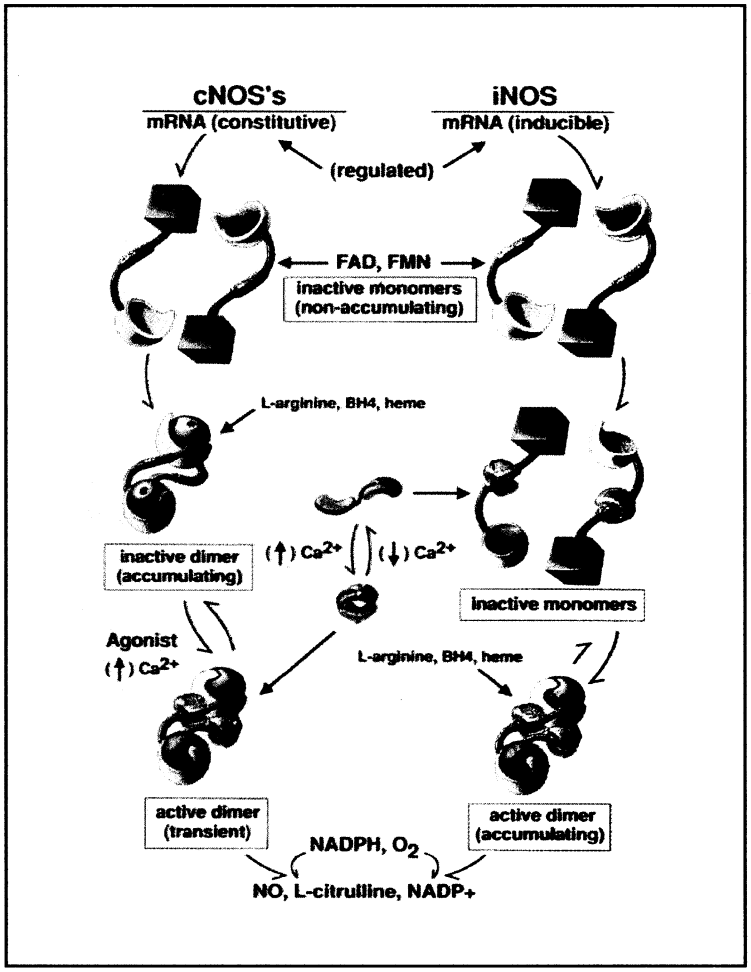
NOS III was first identified in endothelial cells, and therefore named eNOS. Immunohistochemical studies have localized the enzyme to various types of arterial and venous endothelial cells in many tissues, including those in human (Pollock *et al.*, 1993). In addition, NOS III has also been found in syncytotrophoblasts of human placenta (Myatt *et al.*, 1993) and interstitial cells of the canine colon (Xue *et al.*, 1994). This gene is mapped to the 7q35-7q36 region of chromosome 7 (Marsden *et al.*, 1993), and like NOS I, NOS III is constitutively expressed. The gene contains 26 exons spanning about 21 kb of the genomic DNA. The translation initiation and

termination sites are located in exon 1 and exon 26 respectively, leaving the untranslated region of about 22 nucleotides. Unlike NOS I and NOS II, which primarily exist in the cell as cytosolic proteins, NOS III is thought to exist in a membrane-bound form (plasma and microsomal) through a single myristoylation site on the enzyme. Phosphorylation results in detachment of the enzyme from the membrane and subsequent translocation to the cytosol.

#### 1.2.2.3.2 Regulation of activity and NOS III expression

Like NOS I, NOS III is controlled by calmodulin/$Ca^{2+}$, and several mechanisms have been proposed for its regulation. For example, sheer stress produced in the flowing blood was shown to give rise to an acute increase in NO release (Lamontagne *et al.*, 1992), and an upregulation of endothelial NOS expression (Nishida *et al.*, 1992). A putative sheer stress response element in the promoter region of NOS III has since been cloned (Marsden *et al.*, 1993), suggesting increased sheer stress may likely give rise to increased NOS III gene expression and greater capacity for vasodilation. TNF-$\alpha$ has also been shown to down-regulate NOS III mRNA, protein and activity in cultures of bovine endothelial cells (Nishida *et al.*, 1992), and the effect has been attributed to destabilization of mRNA NOS III (Yoshizumi *et al.*, 1993). As in the case of NOS I, there is evidence for stimulatory effects of estrogen on NOS III activity and/or expression.

**Figure 1.2: Crucial differences between NOS isoforms**




Nathan and Xie (1994) Cell: **78,** 915-918

### 1.2.3 Structure of NOS enzymes

NOS are homodimers whose monomers are two enzymes fused. The COOH-terminus is the reductase domain while the $NH_2$- terminus is the oxidative domain. The central portion of the enzyme contains a binding site for $Ca^{2+}$/calmodulin. Generally, constitutive transcription of NOS I and III gives rise to the formation of noninteractive monomers (Fig. 1.2). Upon binding to heme, $BH_4$ and L-ariginine, a conformational change is presumed to take place at the N- terminal region to allow for dimerization of the monomers, such that the oxidase domain of one monomer is approximated with the reductase counterpart of the other monomer (Nathan and Xie, 1994). Such head-to-tail orientation allows interdomain contact without violating constraints imposed by hydrodynamic values, and this would also imply that the homodimer is elongated (Schmidt *et al.*, 1991). It should be noted that dimerization of the two constitutively expressed NOS isoforms does not directly lead to activation of the enzymes. Rather, the binding of $Ca^{2+}$ ions (whose levels increase as a result of agonists) to calmodulin leads to a conformational change in the calmodulin structure and subsequent activation of the protein. The active calmodulin binds to the NOS homodimer at an exposed, hydrophobic site leading to "rotation" of the reductase domain and activation of the (NOS I or NOS III) dimer. In contrast, the basic, hydrophobic site in NOS II monomers can bind calmodulin even at trace levels of $Ca^{2+}$ present in the cytosol of resting cells (Cho *et al.*, 1992). Rotation of the reductase imparted by the binding of calmodulin, however, is not

sufficient for NOS II to be enzymatically active. L-arginine, heme, and $BH_4$ are all required for dimerization of NOS II monomers. Since these positive allosteric regulators are replete in cells transcribing NOS II, neither monomers of NOS II nor calmodulin deficient NOS II dimers are likely to accumulate under normal conditions. The binding affinity of NOS II to calmodulin is sufficiently high that the strong NOS II-calmodulin interaction ensures sustained enzymatic activity, even in the absence of calcium influxes. This property ensures that the enzyme is fully active in the presence of NAPDH, L-arginine, and oxygen (Abu-Soud and Stuehr, 1993). Taken together, the main control for activity of NOS I and III rests in the level of calcium in the cytosol while that for NOS II depends on the level of gene transcription.

### 1.2.4 Major roles of NO

Since the discovery that NO can be produced by a mammalian cell (Stuehr and Marletta, 1985), a tremendous amount of research has been devoted to studying the physiological roles of this molecule. In fact, it was named "Molecule of the Year" by the journal Science in 1992 (Koshland, 1992). Depending on where it is synthesized, the amount produced, the targets and most importantly, its levels in the local environment, NO can exert remarkably different effects. For example, in the endothelium, NO is vital to the regulation of muscle tone (Palmer *et al.*, 1987). In controlling the blood flow, NO is thus indirectly involved in the regulation of

oxygen supply to organs. It also prevents platelet adherence and aggregation as well as neutrophil aggregation to the vascular endothelium (Billiar, 1995). To varying degrees, NO can down-regulate a number of adhesion molecules, namely vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin (CD62E), and P-selectin (CD62P) (Spiecker *et al.*, 1998). Impaired NO synthesis by the endothelium leads to adverse consequences. In both spontaneously hypertensive rats and in humans with essential hypertension, NO production has been shown to be defective (Panza *et al.*, 1990; Durante *et al.*, 1988). Nitric oxide produced by NOS I plays a role as a neurotransmitter in peripheral autonomic neurons referred to as noradrenergic, noncholenergic nerves (Gillespie *et al.*, 1989). Also, it promotes motility of the gastrointestinal tract. In contrast, at higher levels, as when produced by the NOS II, NO functions as an inflammatory mediator making it essential to the host innate response against microbial pathogens especially intracellular bacteria. NO has also been shown to be effective in mounting the response against certain viruses (Croen, 1993; Karupiah *et al.*, 1993), protozoa (Liew *et al.*, 1991), and helminth (Lane *et al.*, 1994). The contributions of NOS II and NO in the immune system in general, and innate immunity, in particular, is discussed in greater details later (Section 1.2.6)

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The ability of NO to inhibit tumor cell growth or promote tumor cell death was the first function of NO to be discovered (Nathan, 1992). NO derived from Mϕ can

### 1.2.5 Mechanisms for NO actions

Nitric oxide is a powerful free radical that can exert its functions via a number of mechanisms. When "attached" to a carrier to extend its short half life, NO can reach target cells by simple diffusion. Being a lipophilic molecule, NO can readily traverse the lipid bilayer and travel to various intracellular locations where it can perform its duty. One of those places is the nucleus where it may distort the structure of DNA by interacting with the amine groups of the nitrogenous bases. However, given that the half-life of the authentic NO gas is less than 15 seconds, NO could carry out its functions much more effectively if it reacted with other radicals to form more stable products. Indeed, it is thought that the chemistry of NO mostly involves interrelated redox forms, the most important ones being those with transitional metal ions and with free thiols, with oxygen-derived free radicals, and with molecular oxygen (Abramson *et al.*, 2001). For example, the binding of NO to prosthetic iron groups and thiol groups forming complexes that can lead to activation or inactivation of many important enzymes. The physiologically most relevant action of NO is the activation of soluble guanylate cyclase by nitrosation of its haem moiety (Ignarro, 1990). Subsequent increase in cGMP levels is crucial in vasorelaxation in the vasculature and neurotransmission in the central nervous system (CNS). In addition to soluble guanylate cyclase, NO is also thought to help activate cyclooxygenase (COX). The inducible type of COX, COX-2, appears to be activated under stimulation with low concentrations of NO, whereas higher

concentrations may inhibit the enzyme. For example, glyceraldehyde-3-phosphate dehydrogenase is inhibited by NO via iron nitrosylation of the thiol group at the active site of the enzyme (Molina *et al.*, 1992). Aconitase, NADH: ubiquinone oxidoreductase and succinate: ubiquinone oxidoreductase of the electron transport chain are inactivated by the attacks on the iron of the iron sulfur (Fe-S) clusters essential to the function of these enzymes (Drapier *et al.*, 1986). In addition to disrupting iron-sulfur clusters and S-nitrosylating, NO can also inactivate proteins by binding to zinc fingers or by peroxidation of membrane lipids (Bogdan *et al.*, 2000). Because of the importance of these molecules in cell function and survival, their inactivation by NO results in cytostasis and cytolysis of invading micro-organisms and tumor cells (Hibbs *et al.*, 1987; Stuehr and Nathan, 1989). Thus, the inhibition of these and other enzymes (*e.g.*, ribonucleotide reductase and cytochrome P450) is believed to be the mechanism by which NO can inhibit the growth of target cells, whether they be invading microorganisms, tumor cells or lymphocytes. The highly active free radical can also react with superoxide anion to form peroxynitrite, nitrogen dioxide and hydroxy radicals. Peroxynitrite is a highly toxic compound which nitrosylates tyrosine residues on proteins leading to accumulation of injurious intracellular oxidants (Pryor and Squadrito, 2001), or a change/disruption of normal protein function (Hausladen *et al.*, 1996). In addition, the ability of peroxynitrite to spontaneously react with other free radicals to generate other ROI and RNI leads to a possibility of DNA mutation. Lastly, in the

presence of molecular oxygen, NO is spontaneously converted to nitrite and nitrate, inactivating the molecule. Nitrite and nitrate are NO indicators in sera and culture media (Stamler *et al.*, 1992). Originally, it was thought that NO actions were strictly limited to the local environment. This is because free radical NO is rapidly consumed or inactivated either when released from the cell, within the cell, or in the circulation where it binds to the heme group of erythrocyte hemoglobin. However, Stamler and colleagues (1992) showed that NO may also form stable adducts with albumin or glutathione in the form of S-nitrosothiols (such as S-nitroglutathione), S-nitrosylated proteins, and nitrosyl-metal complexes which can circulate and diffuse to remote sites, where bioactive NO is spontaneously released (Billar, 1995). Alternatively, NO can also be freed from the complexes upon cleavage by ectoenzymes found on such cells as T and B lymphocytes (Henson *et al.*, 1999). This process may not be limited to extracellular transport of NO as there is evidence for glyceraldehyde-3-phosphate in platelets serving as an intracellular storage and transport vehicle for NO (McDonald *et al.*, 1993). In this light, where NO can be stored and travel long distance without losing its activity, it is conceivable to think of NO as having hormone-like properties.

### 1.2.6 NOS II, NO and their impact on the immune system

Of the three isotypes of the NOS family, it is NOS II that has captured most of the attention within the research community. The special interest is most likely due

to its inducibility and the ability to release a sustained high output of NO. Findings which show that over-expression of NOS II and over-production of NO may contribute to pathogenesis deepen a desire to further our understanding as to how this enzyme is regulated.

Because of its capacity to induce apoptosis, NO might have a place in T cell selection and developmental processes. Epithelial cells and DC in the corticomedullary junction and medulla of the thymus have been shown to constitutively express NOS II whose expression is further enhanced upon contact with self antigens, alloantigens, or with thymocytes activated by TCR stimulation (Tai *et al.*, 1997; Aiello *et al.* 2000, Moulian *et al.*, 2001). The literature shows that double positive thymocytes are highly susceptible to killing by NO whereas single positive thymocytes are not, suggesting that NO released by NOS II expressing stromal cells is among the mechanisms whereby double positive thymocytes are deleted in the thymus (Tai *et al.*, 1997; Aiello *et al.,* 2000, Moulian *et al.*, 2001). Also, because of its capacity to induce apoptosis, nitric oxide has been proposed to confer host protective effects during infectious diseases by inhibiting tissue fibrosis (Hesse *et al.*, 2000).

*In vivo* production of NO and superoxide by phagocytes (Mϕ and neutrophils) in a $CD4^+$ T cell dependent manner is crucial to systemic anti-tumor immunity, as deletion of the NOS II gene and tumor-mediated suppression of macrophage NOS II expression correlate with reduced tumor rejection (DiNapoli *et al.*, 1996).

Conversely, production of NO in certain NOS II-transfected or NOS II upregulated melanoma or sarcoma cells prevents metastasis and induces *in vivo* tumor regression (Bodgan, 2001).

As alluded to in an earlier section, NOS II is in most instances expressed following cell activation by immunologic and inflammatory stimuli. It is the high NO output generated by the inducible isoform (approximately a thousand fold that released by the other two isoforms) that accounts for its role as an inflammatory mediator. Employing the mechanisms discussed above, NO and its intermediates provide a first line host defence against invading pathogens. In fact, RNI (NO and its derivatives) constitute the most important defence mechanism against intracellular bacteria like *Mycobacterium tuberculosis*, one of the most successful pathogens of mankind. The importance of NOS II and NO in innate immunity was revealed from studies where NOS II knock-out mice were more susceptible to infection with intracellular bacteria than wild type mice (MacMicking *et al.*, 1996). In addition, NO also aids in reducing thrombosis formation and improving blood supply to injured tissue.

In addition to the various direct actions of NO discussed here, the antimicrobial activity of NOS II might also be mediated via its indirect functions. Infectious pathogens such as African trypanosomes, *T. Cruzi*, and *Giardia lamblia*, are dependent on exogenous arginine for production of polyamines and cell proliferation. In this light, the induction of NOS II (or arginase) by macrophages and

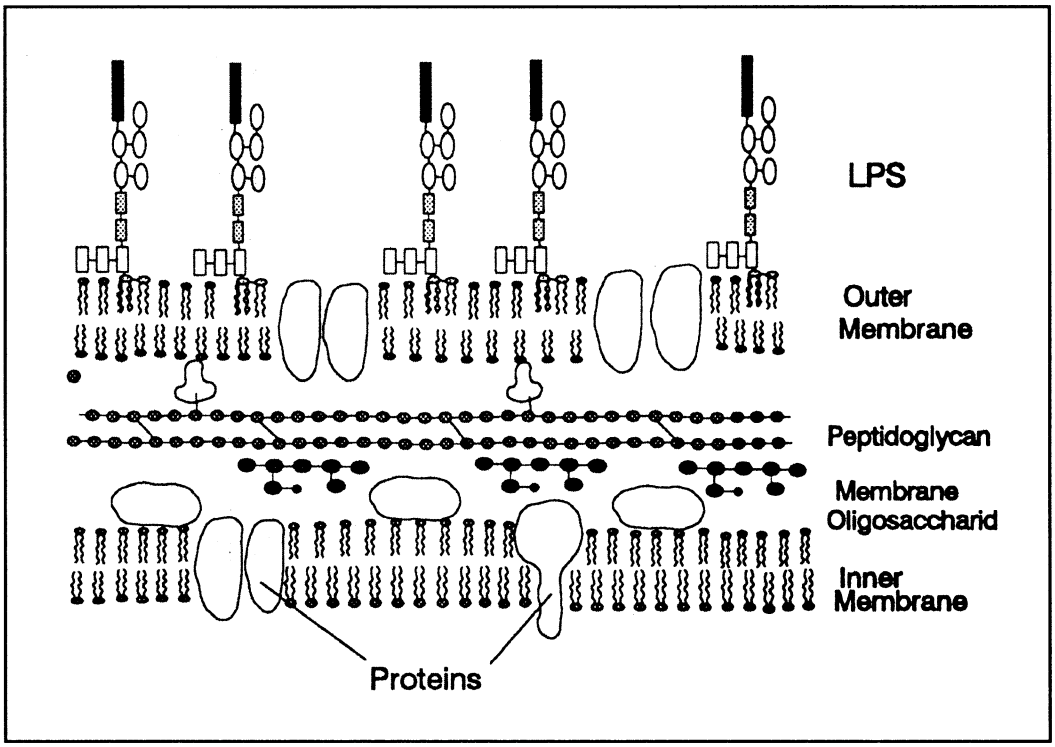
other immune cells will lead to consumption of the available arginine and possible growth inhibition or cell death of the parasites (Eckmann *et al.*, 2000; Piacenza *et al.*, 2001).

However, as much as its actions can benefit the host, excessive NO production can also cause tissue damage to the host's own cells. Therefore, understanding of how NOS II expression can be regulated and hence, how NO production can be modulated is crucial to the development of therapeutic approaches where excessive NO production and/or NOS II over expression have been shown to play an important role in the development and duration of the inflammatory process in a number of chronic inflammatory conditions. This aspect will be further discussed in chapter 6.

## 1. 3. LIPOPOLYSACCHARIDE (LPS)

It is generally accepted that bacterial products are among the most potent stimuli leading to activation of Mo and Mϕ. Of all of those bacteria-derived components which have been studied in the past six decades, endotoxin LPS is considered to be the microbial activator of choice for many studies. The choice is partly because only a small amount of the agent is required for Mo or Mϕ activation (Chen *et al.*, 1992). Adding to the high potency is the fact that highly purified LPS can be readily obtained commercially, and that the component responsible for the biological activity of LPS (see below) has been chemically identified and

**Figure 1.3: *E. coli* envelope organization**




Adapted from Morrison and Ryan (1992)

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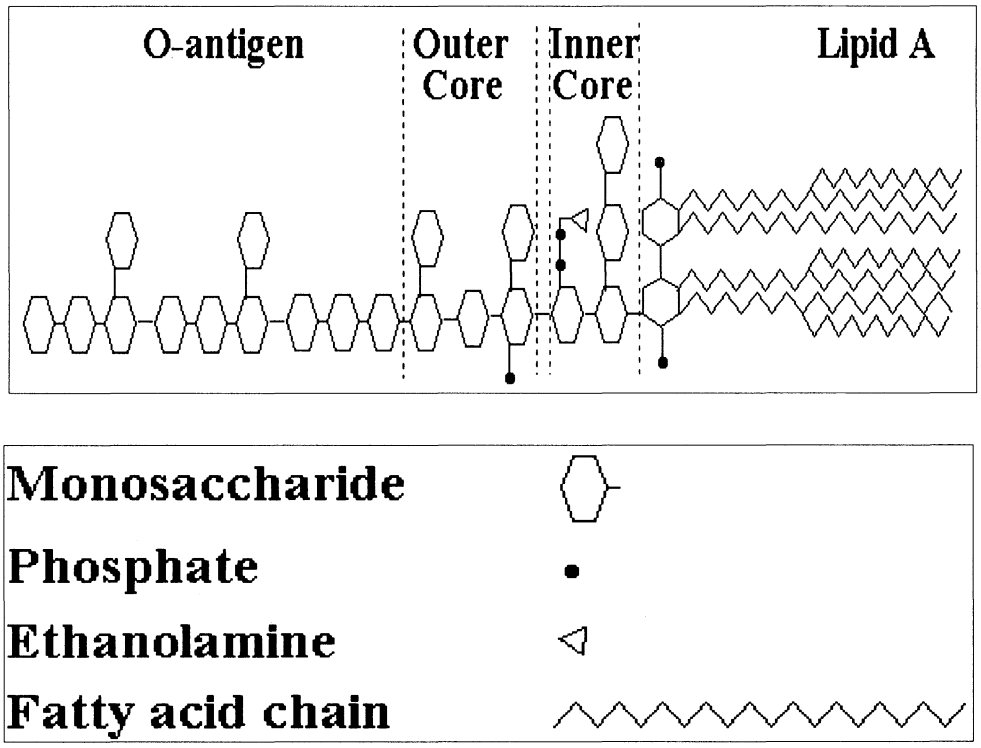
characterized.

### 1.3.1. Structure

LPS constitutes the lipid portion of the outer leaflet of the Gram-negative bacterial cell wall, and is essential for bacterial cell growth (Figure 1.3). The amphipathic LPS molecule consists of an polysaccharide-O chain, a core oligosaccharide and a lipid A moiety.

The O chain is immunodominant, and confers serological specificity to each Gram-negative bacterium. Making up of an inner core and an outer core (Figure 1.4), the oligosaccharide is more conserved than the O chain across the different species of bacteria. The covalently linked lipid A moiety contains a diphosphorylated glucosamine disaccharide acylated with characteristic hydroxylated and non-hydroxylated fatty acid chains. The two phosphate groups are in defined positions. Variation in this structural arrangement such as a reduction of the number of acyl chains or the change in their distribution or degree of saturation results in a dramatic reduction in biological activity (Rietschel et al., 1996). This third region of the LPS molecule is highly conserved in that the moiety is shared by all Gram-negative bacteria (Westphal *et al.*, 1975). The lipid moiety is considered to be the functional essence of the LPS molecule, and not surprisingly the region associated with highest cytotoxicity. Functional significance of lipid A has been documented in a number of studies using different approaches. For

**Figure 1.4: The lipopolysaccharide structure**




Adapted from Morrison and Ryan (1992)

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example, Doe and colleagues (1978) have shown that isolated, highly purified lipid A, under appropriate moiety, will inhibit essentially important LPS-mediated macrophage functions including: (1) production and secretion of cytokines (Chia *et al.*, 1989); (2) generation of procoagulant activity (Niemetz and Morrison, 1977), and (3) macrophage dependent tumor cell cytotoxicity (Doe *et al.*, 1978). Work with synthetic lipid A and other related structures have shown that these components are able to mediate the release of inflammatory cytokines (e.g. IL-1) from Mϕ, further strengthening the importance of lipid A in cell activation (Kotani *et al.*, 1985; Lopponow *et al.*, 1989).

Studies of the chemical requisites for LPS endotoxic activity have shown that the activation of Mo and Mϕ by LPS depends on a novel, peculiar structure of the lipid A moiety present on the invading bacterium (Mueller-Loennies *et al.*, 1998). Recent work has now suggested that biological activities of LPS are determined by the shape of their lipid A portion (Schromm *et al.*, 2000). Only (hexaacyl) lipid A that adopts a conical/concave shape, with the cross-section of the hydrophobic region being larger than that of the hydrophilic region, could induce cytokine production in Mϕ. On the other hand, LPS molecules with cylindrical shape do not exert endotoxic activity on cells, and interestingly these structures have been shown to possess antagonistic activity (e.g. they prevent initiation of cell signalling mediated by biologically active LPS). Thus, synthetic compounds whose structures resemble

that of the cylindrically shaped LPS may provide novel insights for the development of therapeutic approaches for conditions like sepsis.

### 1.3.2 Biological activity

During the course of infection by Gram-negative bacteria, LPS is shed from the outer membranes of the invading pathogen, and released into the circulation, where it subsequently plays a crucial role in initiating the immune response against the micro-organism. There is substantial evidence from both *in vitro* and *in vivo* studies to support a crucial role of LPS in eliciting, in macrophages, most of the cellular responses usually attributable to multifunctional inflammatory cells. For instance, it has been established that in macrophages LPS can induce production of a number of important cytokines (both pro-inflammatory and immunosuppressive), such as IL-1, type I interferons (IFN-$\alpha/\beta$), and TNF-$\alpha$ (Aggarwal *et al.*, 1985). Evidence for LPS mediating release of leukotrienes and arachidonic acid has also been documented (Schade *et al.*, 1987). In addition, LPS isolated from *Helicobacter pylori* has also been shown to induce mitogenic activity, spleen growth, and TNF-$\alpha$ production from mononuclear

initiating factor is bacterial sepsis. It is believed that the ability of LPS to initiate activation of multiple inflammatory pathways, and thus production of various inflammatory mediators, is central to the development of sepsis. Sepsis or endotoxic shock is manifested by the overproduction of pro-inflammatory cytokines such as IL-1, TNF-α, and IL-6 by Mϕ. The condition is characterized by fever, hypotension, leukopenia, hypoxia, acidosis and disseminated intravascular coagulation. If left untreated, multisystem organ failure or even death is not uncommon (Tracey *et al.*, 1986). What started out as a beneficial immune response against the bacterial infection turns into a perpetuating cycle of cell activation and production of inflammatory mediators. In addition to sepsis, bacterial LPS has also been detected in plasma of patients with inflammatory bowel diseases (Caradonna *et al.*, 2000). In these patients, there is an increased production of pro-inflammatory cytokines and chemokines in mucosal tissue and in the circulation.

## 1.4 PROTEIN KINASE C (PKC)

### 1.4.1 Structure

First described by Nishizuka (1984) as a histone protein kinase activated by phospholipids, 1,2-diacylglycerol (DAG) and $Ca^{2+}$, subsequent molecular biochemical studies in the past fifteen years have revealed the presence of multiple isoforms of PKC differing in structure, cofactor requirements and substrate specificity. The PKC superfamily is a group of serine/threonine kinases which play

an integral part in many different signalling systems including those that regulate the activities of ion channels, cytoskeletal proteins, and other molecules involved in cell growth and cell function (Casabona, 1997; Toker, 1998). To date, there are about twelve known members discovered in mammalian cells, and several in non-mammalian cells (Mellor and Parker, 1998). The mammalian PKC are subdivided into three smaller subgroups of conventional (or classical), novel and atypical PKC (Fig. 1.5). The conventional PKCs (cPKC), which include -α (alpha), -β1 (beta 1), -β2 (beta 2), and -γ (gamma), can be activated by phorbol esters, DAG, and $Ca^{2+}$ (Table 1.2). Lacking the $Ca^{2+}$ binding motif seen in the conventional group, the novel PKC (nPKC) group, which is made up of -δ (delta), -ϵ (epsilon), -θ (theta), and -η (eta) (L), does not depend on elevated calcium levels for activation. Rather, they are activated by DAG, phorbol esters and certain fatty acids. The atypical PKCs (aPKC) include -ζ (zeta) and -λ (lamda)/ι (iota) (Ono *et al.*, 1989; Gomperts, 2002) that are neither responsive to phorbol esters nor to $Ca^{2+}$. PKC of the atypical sub-family may have some constitutive activity but are also activated by phosphoserine (PS), phosphatidylinositides or unsaturated fatty acids. It is thought that the lamda isoform is a mouse homologue of the human iota isoform (Nishizuka, 1995). Exactly how these two isotypes are activated has not been fully elucidated, but it has been suggested to involve the Ras protein (Diaz-Meco *et al.*, 1994). In addition to the three subgroups above, there is also a group of PKC-related kinases (PRK), of which there are PKC-μ and its mouse homologue termed PKD, PKCv, or

## Table 1.2: Characteristics of protein kinase C isoforms

| Isoform | Protein size (kDa) | Known activators | Predominant expression |
|---|---|---|---|
| **Conventional PKC (cPKC)** | | | |
| Alpha (-α) | 74 | $Ca^{2+}$, DAG, cis-FA, PS | Universial cells |
| Beta 1 (-β1) | 81 | $Ca^{2+}$, DAG, cis-FA, PS | Fibroblasts |
| Beta 2 (-β2) | 80 | $Ca^{2+}$, DAG, cis-FA, PS | Spleen, kidney |
| Gamma (-γ) | 80 | $Ca^{2+}$, DAG, cis-FA, PS | Rat brain |
| **Novel PKC (nPKC)** | | | |
| Epsilon (-ε) | 97 | cis-FA, $PIP_3$ | Murine brain |
| Delta (-δ) | 82 | DAG, $PIP_3$ | Lung, brain, kidney |
| Mu (-μ)/PKD | 104 | PMA, PKC-ζ | Thymus, lung |
| Theta (-θ) | 79 | ? | Skeletal muscle, T lymphocytes |
| Eta (-η) | 68 | $PIP_3$ | Lung, skin |
| **Atypical PKC (aPKC)** | | | |
| Iota (-ι)/Lamda (-λ) | 67 | ? | Ovary, testis |
| Zeta (-ξ) | 67 | PS, FA, $PIP_2$, $PIP_3$ | Lung, fibroblasts,epidermis |

cis-FA, cis-unsaturated fatty acid; DAG, diacyglyceride; PS, phosphoserine; PIP3, phosphotidyl-inositol 3 phosphate.

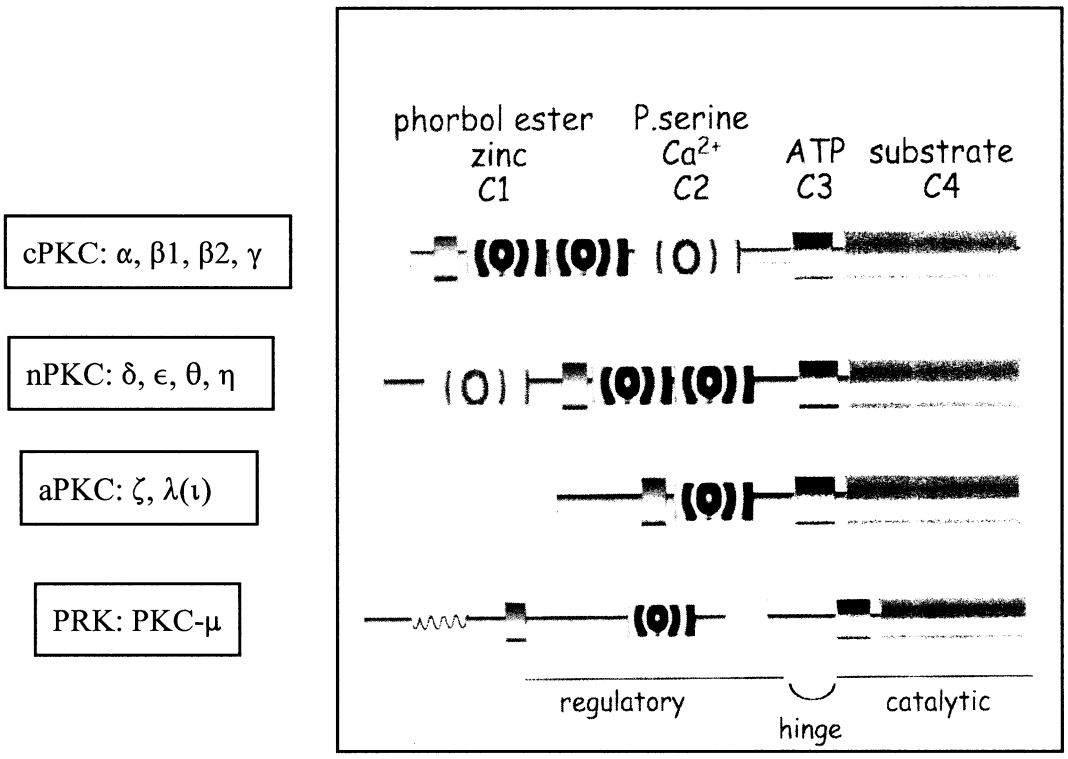
PKD2. Originally categorized in the novel PKC or even considered as an aPKC by some workers, PKC-μ/PKD has now been placed in the PRK group owing to subtle differences in its structure compared to that of the others (see below).

As depicted in Figure 1.5, the structure of PKC isoforms consists of conserved domains (C1-C4) flanked by variable domains (V1-V5), whose function is not clear. The V1-V3 section of the N-terminus makes up the regulatory domain in all PKC isoenzymes, while the V3-V5 portion constitutes the catalytic domain. Separating the regulatory half and the catalytic half of the PKC molecule is the hinge region, located within the third variable region. The hinge is susceptible to calpain ($Ca^{2+}$ dependent neutral protease) digestion. The C1 region contains two cysteine rich zinc finger like sequences, necessary for the binding of DAG and phorbol ester. The crystal structure of the isolated C1 domain of PKC-δ has shown a globular species comprising two-beta sheets, which form the ligand binding cavity (Zhang *et al.*, 1995). The binding of PKC-δ to DAG alters the hydrophobicity of the surface of the C1 domain, thus allowing hydrophobic interactions and membrane targeting (Hurley *et al.*, 1997). Also located in C1 is a pseudosubstrate binding motif, which has an autoregulatory function of blocking the catalytic site located in the C-terminus of the enzyme. Analyses of the crystal structure of the purified C1 domain have shown that the aPKC isoforms have only one of these finger motifs, and thus lack the residues necessary to form the hydrophobic surface at the ligand-binding site (Hurley *et al.*, 1997). These isoforms

**Figure 1.5: The structure of the PKC superfamily**

For clarity purposes, the variable regions are not labelled on this diagram. The structure of PKC is organized as such that each constant region is flanked by two variable regions, whose function is not really known. Each PKC starts with V1 and ends with V5.

Adapted from Gomperts, 2002; p. 121




Adapted from Gomperts, 2002; p. 121

are, therefore, unable to respond to stimulation by either DAG or phorbol ester. Structural analyses of the C2 domain from synaptogamin known to share homology with that of PKC have shown that this region is rich in beta-sheet. Interactions between these anti-parallel sheets forms a novel $Ca^{2+}$ binding pocket, which can coordinate two metal ions (Sutton *et al.*, 1995). As with the C1 domain, PKC isoforms which do not respond to calcium lack the necessary residues to accommodate metal ions. Of the three PKC subgroups, only the conventional PKC isotypes have such required residues to form the cavity. Subsequently, they are the only ones which are responsive to calcium activation. The C2 region also houses part of the receptor for the activated C-kinase 1 (RACK-1) binding domain (Mochly-Rosen *et al.*, 1995).

PKC-μ is structurally different from the others in this group in that the zinc finger like domains are separated by an unusually long stretch of 74 amino acids. The extension forms a putative transmembrane domain and a pleckstrin (platelet and leukocyte C- kinase substrate protein) homology domain (PH domain) (Fig. 1.5). This isoenzyme also does not have the typical pseudosubstrate region and has shown poor binding of phorbol esters. For these reason, PKC-μ is referred to by some researchers as a member of the aPKC subgroup.

The C-terminal regions spanning from C3 to V5, have been established in all PKC isoenzymes as the catalytic domain. C3 contains a consensus ATP-binding sequence, similar to that seen in other kinases. The C4 region constitutes a

## Table 1.3: Phosphorylation sites that render PKC isoforms catalytically competent

| Isoform | In activation loop | | In C-terminus site 1 | | In C-terminus site 2 | |
|---|---|---|---|---|---|---|
| hPKCα | GVTTRTFCGTPDYIAPE | T497 | RGQPVLTPPDQLVI | T638 | QSDFEFGSYVNPQ | S657 |
| HPKCβ1 | GVTTKTFCGTPDYIAPE | T500 | RQPVELTPTDKLFI | T642 | QNEFAGFSYTNPE | S661 |
| HPKCβ2 | GVTTKTPCGTPDYIAPE | T500 | RHPPVLTPPDQEVI | T641 | QSEFEGFSFVNSE | S660 |
| hPKCγ | GTTTRTFCGTPDYIAPE | T514 | RAAPAVTPPDRLVL | T655 | QADFQGFTYVNPD | T674 |
| hPKCδ | ESRASTFCGTPDYIAPE | T507 | NEKARLSYSDKNLI | S645 | QSAFAGFSFVNPK | S664 |
| hPKCι | GVTTTTFCGTPDYIAPE | T566 | REEPVLTLVDEAIV | T710 | QEEFKGFSYFGED | S729 |
| hPKCη | GVTTATFCGTPDYIAPE | T510 | KEEPVLTPIDEGHL | T650 | QDEFRNFSYVSPE | S672 |
| hPKCθ | DAKTNTFCGTPDYIAPE | T538 | NEKPRLSPADRALI | S676 | QNMFRNFSFMNPG | S695 |
| hPKCζ | GDTTSTFCGTPNYIAPE | T410 | SEPVQLTPDDEDAI | T552 | QSEFEGFEYINPL | E579 |
| hPKCλ | GDTTSTFCGTPNYIAPE | T411 | NEPVQLTPDDDDIV | T563 | QSEFEGFEYINPL | E582 |
| hPKCμ | KSFRRSVVGTPAYLAPE | S742 | Absent | | Absent | |

Adapted from Gomperts, 2002; p. 203

Phosphorylation targets are indicated in red. The site 2 serines are substituted by a glutamate (E, yellow) in PKC-ζ and PKC-λ

### 1.4.2 Activation of PKC

PKC is synthesized as an inactive catalytically incompetent protein residing primarily in the cytoplasm (Dutil and Newton, 2000). In order for the kinase to be functional, three phosphorylation processes have to take place sequentially followed by removal of the pseudosubstrate from the catalytic site. Phosphorylation of a threonine (Table 1.3) by a protein kinase (*e.g.* Phospholipid dependent kinase -1 [PDK-1]) in the activation loop within the catalytic domain leads to auto-phosphorylation of the two C-terminus sites. The enzyme is now still inactive but catalytically competent because of the presence of the pseudosubstrate in the catalytic site. When only C1 or C2 ligand is present, the substrate-binding site is assumed to maintain contact with the pseudosubstrate region, keeping the enzyme catalytically competent but inactive. The calcium-binding site is thought to interact with phospholipid molecules like PS, increasing the affinity of the enzyme for the phospholipid. Subsequent binding of co-factors DAG, $Ca^{2+}$ and phospholipids (Table 1.2) typically induces a conformational change to the catalytic domain allowing for the removal of the pseudosubstrate, and thus making the once-occupied site now accessible to the real substrate.

Studies using synthetic peptides have revealed a basic consensus phosphorylation motif of RXXS/TXRX, where X represents any amino acid, and the importance of basic residues (*e.g.*, Arg [R]) N- and C-terminal to the

phosphoacceptor residue (Toker *et al.*, 1998). Although there are some subtle differences between the three subgroups, all PKC isoforms preferring basic residues at positions -6, -4 and -2 to the Ser/Thr with cPKC also prefering basic amino acids at positions +2, +3 and +4. As discussed previously, the N-terminal extension of PKC-μ (Fig. 1.5) is thought to be possibly responsible for its strikingly distinct optimal phosphorylation motif from all other PKC. This particular isoform has a strong selectivity for Leu at the -5 position (Nishikawa *et al.*, 1997). Taken together, despite the subtle difference, it appears that members of the PKC family tend to phosphorylate very similar substrates. This suggests that there must exist other mechanism(s) which can dictate which PKC isoforms participate in what signalling pathways. Several switch points can come into play here, and these can include different cofactors as discussed above, and cellular localization. It is now known that individual PKCs are brought to appropriate subcellular sites (*e.g.*, plasma membrane, nuclear membrane) where their substrates are located by PKC binding proteins including receptors for activated C kinase (RACKs) (Schechtman and Mochly-Rosen, 2001) and proteins interacting with C kinase (PICKs) (Gomperts, 2002). The anchors, which have shown some selectivity/specificity among various PKC isotypes (e.g. RACK1 is specific for the beta-2 isoform while RACK2 is the PKC-ε chaperone protein), also help appropriately position PKC to respond to specific receptor mediated activating signals. The activated PKC is then translocated to other subcellular locations where substrates of the activated PKC

are within the vicinity.

### 1.4.3 Expression and functions of the individual PKC isotypes

The importance of PKC in pathophysiology and signalling mechanisms is not a novel concept. However, what it is less clear is the exact mechanisms used by these enzymes to exert their effects. Adding to the complexity of the picture is the fact that not only may the same PKC isoenzymes be expressed in different cells but that several PKC isoforms may be present within the same cell type. It is, therefore, difficult to fully understand what each of these PKC isoforms does at a particular moment in a given cell. The story becomes even more complicated when one has to reconcile the fact that a given PKC isoform may be activated by different PKC(s) in different cell types in response to different stimuli. For example, in epithelial cells, PKC-mu/PKD is activated by the upstream PKC-eta (Brandlin *et al.*, 2002), but in aortic smooth muscle cells PKC-mu needs PKC-delta for its activation (Tan *et al.*, 2002).

Below is a brief review of their expression characteristics and some of their known functions. It should be kept in mind that the summary is by no means a complete list of functions for the various PKC isotypes. Whenever possible, the review will be focussed on regulation of individual PKC isoenzymes in Mo and Mϕ functions.

### 1.4.3.1 Conventional PKC

#### 1.4.3.1.1 PKC-alpha (PKC-α)

PKC-α appears to be a ubiquitous enzyme which can be found in most or all tissues. In human monocytic cell lines, this isoform is thought to be required for superoxide production by activated cells (Li *et al.*, 1999), and for the inhibition of Fc-γ receptor mediated intracellular killing of *Staphylococcus aureus* by human Mo (Zheng *et al.*, 1995). In line with its role as a positive regulator of production of ROIs, PKC-α is also believed to play a role upregulating transcription of pro-inflammatory mediators such as IL-12, G-CSF, and NOS II (St-Denis *et al.*, 1998).

#### 1.4.3.1.2 PKC-beta (PKC-β)

This isoenzyme consists of two related forms, PKC-β1 and PKC-β2. They are a product of alternative mRNA splicing. Like PKC-α, PKC-β is typically expressed in most tissues. However, unlike the alpha isoform, PKC-β is responsible for the stimulation of the Fc-γ receptor mediated killing of *S. aureus* (Zheng *et al.*, 1995). In addition, there is also evidence to support a role of both PKC-β and PKC-δ in the differentiation of myeloid leukaemia cells into Mϕ (Kaneki *et al.*, 1999).

#### 1.4.3.1.3 PKC-gamma (PKC-γ)

Compared to the other members of the PKC family, PKC-γ has the most

restricted tissue expression profile. The enzyme is only found in the CNS (Nishizuka, 1988). Although not expressed during early embryonic and fetal development of the nervous system, PKC-γ is present in great abundance in adult CNS (Sposi *et al.*, 1989). Its preferential expression in the nervous system is thought to play a role in cellular mechanisms through which pathological brain activity impairs certain aspects of spatial memory (Beldhuis *et al.*, 1992).

### 1.4.3.2 Novel PKC

#### 1.4.3.2.1 PKC-delta (PKC-δ)

Both delta and epsilon isoforms have a rather broad expression profile across the tissues. In addition to its role in monocyte differentiation, the delta and epsilon isoforms have been associated with the signalling pathways leading to apoptosis (Sawai *et al.*, 1997). Work with rottlerin, a PKC-δ specific inhibitor, by Kontny *et al.* (2000) has shown that suppression of PKC-δ activity resulted in inhibition of DNA binding of such transcription factors as AP-1 and NF-κB leading subsequently to attenuation of IL-1 and TNF-α production by human monocytic cells previously exposed to LPS or PMA.

#### 1.4.3.2.2 PKC-theta (PKC-θ)

Unlike other members of the PKC superfamily, PKC-θ has a limited tissue

distribution. The kinase is predominantly expressed in hemapoietic cells (particular T cells) and, skeletal muscle. To a lower extent, this particular isoform may also be found in lung, skin, and brain (Osada *et al.*, 1 992). Sun and colleagues (2000) have recently shown that PKC-θ is rapidly recruited to the site of TCR clustering and that the kinase is required for TCR-induced NF-κB activation in mature T cells, but not in developing thymocytes. In a number of T cell clones and in primary lymph node T cells from TCR transgenic mice, ten minutes is all that required to observe the translocation of PKC-θ to the plasma membrane following T cell recognition of the antigen.

#### 1.4.3.2.3 PKC-epsilon (PKC-ε)

The epsilon isoform is found in great abundance in brain and other (neuronal) tissues (Toker, 1998). As with other isoforms in the novel PKC subfamily, PKC-ε is responsive to both DAG and PS *in vivo*. There is also evidence for its activation by the phosphotidylinositol-3-kinase (PI-3K) lipids (PtdIns-3,4,5-P3) *in vitro* (Toker *et al.*, 1994), as well as mitogenic stimuli *in vivo* (Ohno *et al.*, 1994). When overexpressed in fibroblasts, PKC-ε can function as an oncogene. Increased activity of the epsilon isoform has also been associated with characteristics of neoplastic transformation (e.g. increased growth in soft agar and tumor formation in nude mice) (Cacace *et al.*, 1993). In addition, this PKC isoform has been linked to the activation of a number of transcription factors, namely c-fos and c-jun, NF-AT,

AP-1, and NF-κB.

#### 1.4.3.2.4 PKC-eta (PKC-η)

In normal conditions, PKC-η is thought to be most abundantly expressed in the skin and lung, but only slightly in the thymus and the brain (Bacher *et al.*, 1991). Both the alpha and eta isoforms are potent activators of Raf-1. Using transfection and antisense approaches in glioblastoma cell lines, Hussaini *et al.* (2002) have demonstrated that PKC-η can help confer cell resistance to UV-and gamma radiation-induced apoptosis by preventing activation of caspase-9.

Confocal analyses of mammary epithelial cells MCF7 and HEK293b has revealed that PKC-η co-localizes with PKC-mu/PKD in perinuclear compartments, and the former directly phosphorylates the latter (Brandlin *et al.*, 2002). The observation suggests that PKC-η is crucial for PKC-μ dependent cellular processes. Interestingly, when eta and mu isoforms are co-expressed, PKC-μ mediated effects are enhanced while, at the same time, those triggered by PKC-η will be reduced. Specifically, activation of PKC-μ by PKC-η leads to an increase in the PKC-μ dependent p42/p45 MAPK cascade and a simultaneous decrease in the PKC-η triggered JNK signalling pathway (Brandlin *et al.*, 2002). Findings such as these suggest a mutual regulation for these two members of the PKC family affecting different arms of the p38/ERK/JNK MAPK pathways.

### 1.4.3.3 Atypical PKC (aPKC)

#### 1.4.3.3.1 PKC-iota (PKC-ι)/PKC-lambda (PKC-λ)

PKC-ι has been shown to protect cells against drug-induced apoptosis. Par-4, a gene which is induced during apoptosis, specifically interacts with and potently inhibits the kinase activity of members of the aPKC subgroup. This suggests a role for PKC-ι and the other aPKC in the regulation of cell growth (Diaz-Meco *et al.*, 1996).

#### 1.4.3.3.2 PKC-zeta (PKC-ζ)

PKC-ζ is another member of the PKC superfamily that is ubiquitously expressed in most tissues. In the past decade, evidence has been put forward to support a role for PKC-ζ and its close relative, PKC-λ as crucial mediators of mitogenic signal transduction. The zeta isoform is thought to be required for maturation of Xenopus oocytes, and for synthesis of DNA in fibroblasts as a consequence of Ras activation (Berra *et al.*, 1993). A number of studies have shown that activation of the mitogen activated protein kinase (MAPK) pathway is downstream of the zeta isoform. For example, co-transfection experiments with PKC-ζ and MAPK have revealed that the serine/threonine kinase stimulated the activation of both MAPK-kinase and MAPK (Berra *et al.*, 1995). However, it should be noted that the activation of MAPK is not restricted to PKC-ζ, but that other PKC family members, including conventional

PKC, are also capable of activating MAPK *in vivo*, demonstrating redundancy among the PKC isoenzymes. Furthermore, PKC-ζ is also implicated as an important mediator of some of the physiological actions of insulin. Stimulation of adipocytes with insulin leads to activation of the kinase in a PI-3K dependent manner (Bandyopadhyay *et al.*, 1997; Mendez *et al.*, 1997). The process of insulin-stimulated glucose uptake is mediated by GLUT4, a major transporter, which acts downstream of PKC- ζ (Standaert *et al.*, 1997). Thus, the zeta isoform might regulate glucose homeostasis in part by mediating translocation of GLUT4. There is also evidence for a requirement of PKC-ζ for Mϕ differentiation in insulin-like growth factor I stimulated cells.

As for a role of PKC-ζ in neoplastic transformation, the picture is less clear in that this PKC form appears to have both growth-promoting and growth-inhibitory properties (Toker, 1998). It is difficult to reconcile such idea for a molecule but recent evidence suggested a rationale for the observation. Kamper *et al.* (1998) proposed the idea of a hierarchical signalling cascade, which involves consecutively the lambda, epsilon, and zeta isoforms in transcriptional activation of the c-fos promoter in cells expressing oncogenic Ha-ras (Kampfer *et al.*,1998). Thus, depending on the expression profile of PKC in the cell of interest, PKC-ζ may function as a growth promoter or growth inhibitor.

#### 1.4.3.3.3 PKC-mu (PKC-μ)/PKD

PKC-μ is the human analogue of PKD, and has been found to selectively associate with PKC-η. PKC-μ is ubiquitously expressed, and its expression is abundant in the thymus and lung (Rennecke *et al.*, 1996). The mu isoform is potently and rapidly activated by a number of agonists including bradykinin, bryostatin and phorbol esters. These stimuli can also activate other members of the PKC family *in vivo*. Although direct phosphorylation of PKC-ζ by the epsilon and eta isoforms has not been clearly established, Abedi *et al.* (1998) demonstrated that activation of PKC-μ in cells occurs via a PKC-dependent pathway, such that PKC-ε and PKC-η act upstream of PKC-μ. Obviously, more work needs to be done before one can decipher exactly how this enzyme regulates *in vivo*, and whether there is also a hierarchical signalling cascade like that observed in PKC-ζ. As mentioned previously, PKC-μ does not have a pseudosubstrate region *per se,* however, mutagenesis studies revealed that deletion or mutation of the PH domain (Fig. 1.5) results in constitutive activation of the enzyme (Iglesias and Rozengurt, 1998). However, it is still unclear as to what co-factors, lipids or proteins or both, bind to the PH domain *in vivo* to result in such activation. Lastly, there is also evidence for a role of PKC-μ in B cell receptor signalling, and its activation following cross-linking of the B cell receptor (Sidorenko *et al.*, 1996).

## 1.5 RATIONALE AND OBJECTIVES OF PRESENT INVESTIGATIONS

Through its action on monocytic cells, bacterial LPS or endotoxin can trigger responses that can be both protective and injurious to the host. As discussed previously (Section 1.1.3), many PAMPs including LPS, peptidoglycan, lipoteichoic acid, lipopeptides, and CpG DNA are all able to activate Mϕ to produce cytokines. Among these, however, only LPS has so far been shown to trigger NOS II expression in macrophages. Although the induction of NOS II by LPS has been fairly well studied and established in murine monocytic cells, much less work is done for the human cells. The main reason is due to the difficulty in inducing *in vitro* expression of NOS II in human monocytic cells using LPS. Over the years, several concepts have been put forward as possible explanations for the peculiar behaviour (Chapter 3) but the fundamental question remains: "what is it about human monocytic cells that is so different from their murine counterparts which causes such different response to LPS" ?. The herein thesis work was, therefore, aimed at trying to answer this question. Understanding of how human NOS II expression in Mo/Mϕ may be regulated would offer important insights, and perhaps potential therapeutic application for inflammatory diseases known to be associated with the over-expression of NOS II and/or over-production of NO, including rheumatoid arthritis (RA) and insulin-dependent diabetes mellitus (IDDM). To help answer the previously posed question, the following investigations were

sequentially conducted. First, we evaluated the ability of human and murine monocytic cells from various cell lines to respond to LPS or phorbol myristate acetate (PMA) (Chapter 3), and produce NO. The assessment was also to confirm the inability of the human cells to make NO following these stimulation. Second, in light of earlier evidence from our laboratory (Mouland, 1998) as well as that from others (Paul *et al.*, 1995; Sodhi and Kumar, 1994) demonstrating that PKC inhibitors could down-regulate LPS-induced NO production in murine monocytic cells, we performed for the first time a complete comparative analysis of PKC isotype expression in human and murine monocytic cells (Chapter 5). Third, the data obtained from such analysis subsequently enabled us to propose, and test a hypothesis that a missing member of the PKC family in human monocytic cells may be responsible for their inability to express NOS II and make NO following LPS stimulation. Fourth, the findings obtained from the third investigation in the cell lines allowed us to test the applicability of the *in vitro* data to *in vivo* situations. To this end, we have tested the hypothesis in monocyte-derived macrophages (MDM) and corresponding plasma samples of patients with rheumatic diseases including rheumatoid arthritis (RA) and spondyloarthropathies. In addition, as aside mini-project but also highly relevant to the main objectives of this thesis, we were interested in investigating the effects of one natural and two synthetic derivatives of Trypterygium *wilfordii Hook* f (TwHf), a Chinese traditional medicine used in the treatment of a number of inflammatory diseases including RA, on the expression of

NOS II, as well as production of NO, TNF-α, and MMP in monocytic cells.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 CHEMICALS

1) Most chemicals for preparation of buffers used in electrophoresis (DNA and protein) and Western blotting were obtained from Sigma Aldrich (Ontario, Canada).
2) LPS (from *E. Coli*, 026:B6, Cat # L2654) was purchased from Sigma Aldrich.

### 2.1.2 CELL CULTURE REAGENTS

All cell culture materials were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). Reagents for bacterial cell cultures were obtained from Invitrogen Life Technologies and Sigma. Cultures were routinely grown in 75-$cm^2$ flasks from Falcon (distributed by Becton, Dickinson, ON, Canada). Depending on the type of experiment being carried out, cells may have been plated on 6-well, 24-well or 96-well microtiter plates (Falcon). In experiments where RNA or proteins were to be extracted, the cells were plated on 35-mm or 60-mm petri dishes.

#### 2.1.2.1 Cell lines

J774A.1, THP-1 and U937 were obtained from American Tissue Culture Collection (ATCC) (Rockville, MD, USA). Raw 264.7 (ATCC TIB 71) was a generous gift from Dr. Ratnam (Department of Public Health, Newfoundland, Canada) and Mono Mac 6 (MM6) was purchased from DSM (German Collection of

Microorganisms and Cell Cultures, Germany).

J774A.1 (ATCC TIB 67) was adapted to culture from a tumor which arose in a female BALB/c mouse in 1968. This cell line was established from the ascites of a tumor induced in a male mouse by intraperitoneal injection of Abelson leukemia virus. Like J774A.1, the Raw 264.7 cell line was established from a tumor induced by Abelson murine leukemia virus in a male BALB/c mouse. The THP-1 cell line (ATCC TIB 202) was adapted from peripheral blood mononuclear cells (PBMC) isolated from a one-year old boy with acute monocytic leukemia. The MM6 cell line was prepared from monocytic cells of an adult with monocytic leukaemia. The U937 cell line, derived from the pleural effusion of a patient with diffuse histiocytic lymphoma, consists of fairly mature monocytoid cells. These cells have many macrophage markers including complement receptor-3 (CR3), Fc receptor-1 (FcR1), non-specific esterase, lysozyme, and peroxidase (Zembala and Asherson, 1989).

Murine macrophage-like cell lines Raw 264.7 and J774A.1 were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, and 7.5% (w/v) $NaHCO_3$. Human monocytic cell lines MM6 and U937 were cultured in the above media plus 9 µg/mL bovine insulin (I-1882, Sigma), 1 mM sodium pyruvate, and 1 mM MEM amino acids. Medium for the THP-1 cells was the same as that for the other two human cell lines (MM6 and U937) except that it also had $2 \times 10^{-6}$ M β-mercaptoethanol (2-ME).

Murine fibroblast cell line L929 was also obtained from ATCC (Cat#: CCL-1). L929 cells were propagated in RPMI-1640 (5% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, 25 mM N-2-Hydroxyl piperazine-N-2-ethane sulphonic acid (HEPES).

### 2.1.2.2 Cytokines

Human recombinant IFN-γ (IF002) was purchased from Chemicon (Temecula, CA, USA). Mouse recombinant IFN-γ was kindly provided by Dr. Mookerjea (Memorial University, Newfoundland, Canada) and human recombinant TNF-α was a generous gift from Dr. Grant (Memorial University).

## 2.1.3 MOLECULAR BIOLOGY REAGENTS

### 2.1.3.1 For DNA and RNA purification

All reagents for plasmid preparations including CONCERT high purity plasmid purification system (Cat # 11451-010) and TRizol were purchased from Invitrogen Life Technologies. Chloroform and other miscellaneous chemicals (molecular miology grade) were obtained from Sigma Aldrich.

### 2.1.3.2 For cell transfection

LipofectAMINE (0.747 mM) and Opti-MEM Reduced Serum Media were

purchased from Invitrogen Life Technologies.

### 2.1.4 ANTIBODIES

#### 2.1.4.1 Primary antibodies

1) Rabbit polyclonal (-β1, -β2, -γ, -δ, -ε, -η, -θ, -ι) and mouse monoclonal (-α) anti-PKC antibodies were kindly provided by Dr. Nigel Groome (Oxford, UK). The concentrations used varied among the different antibodies, but they were typically in the range of 1:1000-1:5000.

2) Rabbit polyclonal PKC antibodies specific to ζ and μ isoforms were from Santa Cruz (Santa Cruz, CA, USA).

3) Rabbit polyclonal anti-NOS II antibody (sc-651) and blocking peptides (sc-651 P) to PKC-η and NOS II were also purchased from Santa Cruz. The antibody was used at 1:800 dilutions, while the peptide at five times higher concentration.

4) Mouse monoclonal IgG2a anti-human TLR4 antibody (0.5 μg/μL, Cat # 14.9917) was purchased from eBioscience (San Diego, CA, USA), and used at 1:50 dilution.

5) Mouse APC-conjugated anti-human TLR-4 antibody was a generous gift from Chemicon, and used at 1:100 dilution.

#### 2.1.4.2 Secondary antibodies

1) Sheep anti-rabbit and anti-mouse horse radish peroxidase-labelled antibodies were from Amersham International (Arlington Heights, IL, USA). The working

concentration was 1:3000.

## 2.2 METHODS

### 2.2.1 DESIGN OF HUMAN (AND MURINE) NOS II-SPECIFIC PRIMERS

In both murine and human, the NOS II sequence is different from that of the NOS I and NOS III in that it only shares 73% and 77% homology, respectively (values obtained from gene alignment using BLAST 2). Also, human hepatocyte and murine monocytic NOS II sequences share 80% homology (Gellar *et al.*, 1993). Taken together, it was necessary to design a primer set that would only allow amplification of the NOS II gene in both human and murine monocytic cells. As the first step then, alignments of sequences retrieved from the National Institute of Health genetic sequence database, GenBank, were performed. Sequence alignments were done using the Basic Local Alignment Search Tool (BLAST)/ BLAST2, while primer design was done with the help of Primer 3 and Omega softwares. In this instance, the design fulfilled the six standard PCR primer criteria. First, primers must not be homologous to other portions of the DNA or to the primer partner, as this would result in primer-dimers formation. Second, the primer must not be self complementary thus eliminating the possibility of a stem loop structure formation. Third, the GC content must be at least 50-60%. Fourth, the primers should have similar melting temperatures, ideally within 5 $^{0}$C of each other. Fifth,

each primer must have at least one G or C clamp at the end of the primer. Sixth, the primer length should not be less than 19 or more than 27 bases.

The designed primers were custom made by Invitrogen Life Technologies. Working solutions of 10 µM were prepared, and stored along with the stock solutions at -20 $^0$C until used.

### 2.2.2 CELL CULTURE

All cell cultures were done at 37 $^0$C in a humidified atmosphere with 5% $CO_2$. Monolayers of J774A.1 and Raw 264.7 were washed twice with phosphate buffered saline (PBS), and the adherent cells were gently scraped off the surface using a sterile rubber policeman. Cell suspensions of THP-1, U937 and MM6 were also washed with PBS. Cell pellets collected after centrifugation were resuspended in medium appropriate for the cell line, and either used for the experiment or re-seeded. Cells were passaged (1:4 splitting) every three days. Only those with viability above 85%, as determined by Trypan Blue exclusion, were used in our studies.

### 2.2.3 PREPARATION OF MONOCYTE-DERIVED MACROPHAGES (MDM) FROM PERIPHERAL BLOOD

Fresh venous blood collected in ACD vacutainers (yellow top) was typically

processed within 2-3 h of collection. The sample was centrifuged for 10 min at room temperature (RT) at 400 x g (Sorvall RT6000: DuPont, New Hampshire, USA). Plasma was transferred to 1.5-mL centrifuge tubes and stored at -70 $^{0}$C for measurements for NO. The cells were diluted 1:1 with PBS, and slowly layered on top of a Ficoll-Paque Plus (Pharmacia, AL, USA) in a 2:1 ratio, and care was taken to avoid mixing of the two solutions. The cells were centrifuged for 40 min at RT at 400 x g. The upper top Ficoll layer was discarded, and the interphase containing PBMC, was transferred to a 50-mL centrifuge tube. The cells were washed with at least 30 mL PBS, and centrifuged for 10 min at 100 x g at RT. The supernatant was removed, the cells washed with 10 ml PBS supplemented with 1% FBS, and then again centrifuged at RT for 10 min at 250 x g. Any red blood cell (RBC) contamination was eliminated by incubating the cell pellet with 5 mL of 0.4 M $NH_4Cl$ for 10 min at RT. PBS (10 mL) was added, and the cell suspension spun down for 10 min at 400 x g. The cell pellet was then resuspended in 10 mL of RPMI-1640 media (10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM MEM amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 25 mM HEPES, and 3 x $10^{-5}$ M 2-ME), counted, and cultured for 4 h. Non-adherent cells were subsequently washed twice with PBS. Adherent monocytes were allowed to differentiate into MDM in culture for 5 days prior to further experimentation.

## 2.2.4 CELL VIABILITY ASSESSMENT

### 2.2.4.1 Trypan Blue Exclusion

The Trypan Blue dye is actively excreted by viable cells, which thus will appear colourless when observed under the microscope. Dead cells will appear blue due to dye up-take. Cell counting was done using a Neubauer improved haemacytometer. Cell viability was determined by the ratio of the number of viable cells counted and the total number of cells (dead plus viable cells).

### 2.2.4.2 MTT Assay

This assay, also known as succinate dehydrogenase inhibition (SDI) test, was used to measure cell cytotoxicity after being exposed to a drug compound. The principle of the assay is based on the reduction of the (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide tetrazolium) (MTT, Sigma) salt by the succinate-tetrazolium reductase of the mitochodrial respiratory chain in metabolically active cells. Therefore, this bioassay detects only viable cells. MTT is cleaved to form a purple coloured water-insoluble formazan salt, which absorbs maximally at 540 nm (Bradshaw *et al.*, 1998). Following the 24-h recovery period after drug exposure, the media was removed from the cells from each well, including blanks, and 100 µL of media containing 0.5 mg/mL MTT was added. The plate was then incubated at 37 $^{0}$C for 4 h. After which time, cell media was removed and the insoluble formazan salt

was dissolved in 100 µL of DMSO (Sigma) with gentle agitation for 20 min. The absorbance was then read at 490 nm on a microplate reader (Model 3550: BioRad, San Diego, CA, USA).

### 2.2.5 TRANSFORMATION AND BACTERIAL CELL CULTURE

20 µL DH5α *E. Coli* competent cells (kindly provided by Mr. Suresh Belkhode, Memorial University) was added to a chilled 0.6-mL microfuge tube followed by 0.3 µL of appropriate plasmid DNA. The control tube contained only DH5α cells. The samples were mixed gently and kept on ice for 30 min. After which time, the tubes were transferred to a 42 $^{0}$C water bath for exactly 2 min and then immediately transferred to ice for another 2 min. Luria LB broth (400 µL, [25 g/L, Invitrogen Life Technologies]) was then added, and the tubes placed in a controlled environment incubator shaker (New Brunswick Scientific Co. Inc, New Jersey, USA) at 37 $^{0}$C for 2 h. Subsequently, 10-100 µL of the LB broth was removed from the tubes and the cells streaked on a solid agar plate (previously equilibrated to 37 $^{0}$C) containing 50 µg/mL ampicillin (Novopharm, Toronto, Canada). Ampicillin was used because the desired plasmids (pBABE and pks1.PKC-η) contained the ampicillin resistance gene $Amp^{R}$. The plate was placed in a humidified 37 $^{0}$C incubator (Fisher) for overnight. Colonies were developed after 14-16 h incubation. A single colony was removed, and placed in 3 mL LB broth containing 20 µg/mL ampicillin. The cells were next cultured for 13 h. The

next day, 1.5 mL of this LB broth was used to prepare a DNA mini-preparation.

## 2.2.6 PLASMID PURIFICATION

### 2.2.6.1 Miniprep

A miniprep was prepared as follows. The broth described above (1.5 mL) was transferred to an eppendorf tube and spun at 4 $^0$C for 2 min in a microfuge (Eppendorf, Centrifuge 5415 C, Brinkmann, USA). The supernatant was removed and 100 µL of freshly made suspension buffer (25 mM Tris, 50 mM glucose, 10 mM EDTA, pH 8.0) was added to the cell pellet. The sample was pipetted up and down several times to ensure complete dispersion of the cells. The tube was then placed on ice, to which 200 µL of freshly made lysis solution (0.1 M NaOH, 1% sodium dodecyl sulphate [SDS]) was added. The sample was gently mixed and incubated on ice for 5 min. Next, 150 µL of a neutralizing solution (3 M potassium acetate, 11.5% [v/v] glacial acetic acid) was added, and the sample was incubated for another 7 min. It was next centrifuged at 10000 x g for 2 min in a microfuge. To the supernatant, 3 µL of 10 mg/mL bovine pancreatic DNAse-free RNAse A (Sigma) was added, and the sample further incubated for 25 min at 37 $^0$C. Subsequently, it was extracted with 1 mL phenol: chloroform: isoamyl alcohol (25:24:1, Sigma), and then re-extracted with 1 mL chloroform: isoamyl alcohol (Sigma). The sample was spun at 10000 x g for 2 min and DNA, present mostly in the aqueous phase, was

precipitated with 2 volumes of absolute ethanol and one-tenth volume of 3 M potassium acetate (pH 8.0). The sample was centrifuged for 20 min at 4 $^0$C, the supernatant decanted, and the pellet washed with 70% ethanol. The air-dried pellet was resuspended in water, and stored at -20 $^0$C until used.

### 2.2.6.2 Midiprep

After identity of the plasmid DNA obtained from the miniprep was confirmed by digestions with restriction enzymes, 10 µL of the original bacterial culture was added to a sterile conical flask containing 20 mL of LB broth supplemented with 20 µg/mL ampicillin. The cells were cultured for 10 to 13 h or when the cell density was approximately $10^9$ cells/mL (1-1.5 $A_{600}$ units/mL). Plasmid DNA used in transfection studies was purified using CONCERT high purity plasmid purification system. All buffers used in the midiprep were provided with the purification system by the manufacturer. Centrifugation was done at 15,000 x g in a Beckman centrifuge (model J-21), unless otherwise stated. Overnight culture (15-20 mL) was centrifuged for 10 min (10,000 x g) at RT. Supernatant was removed carefully but as thoroughly as possible, and the cell pellet resuspended in the RNAse -containing cell suspension buffer (4 mL) until homogeneous. Subsequently, the cell lysis buffer (4 mL) was added, the tube inverted five times to mix, and the sample incubated for 5 min at RT. The lysis was stopped with neutralization buffer (4 mL),

and the mixture left undisturbed for 10 min at RT. The supernatant was loaded onto the provided purification column that had been previously equilibrated with 10 mL of the equilibration buffer in the kit. The solution in the column was allowed to drain by gravity flow. The flow-through was discarded, and the column washed twice with 10 mL each of the wash buffer. The DNA was next eluted from the column with 5 mL of the elution buffer. The solution was again drained by gravity flow. Isopropanol (3.5 mL) was added to the eluate, the sample mixed and centrifuged for 30 min at 4 $^0$C at 15,000 x g. The plasmid DNA pellet obtained was washed with 3 mL of 70% ethanol, and centrifuged for another 5 min at 4 $^0$C. The ethanol wash was carefully and fully pipetted off, and the pellet air-dried for 10-15 min. The DNA was dissolved in 200 µL Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and stored at -20 $^0$C until used in transfection experiments.

### 2.2.7 TOTAL RNA EXTRACTION FROM MONOCYTIC CELLS

Human and murine cells were plated at a density of 1 x $10^6$ per 60-mm dish for overnight at 37 $^0$C (5% $CO_2$), and then exposed to 1 µg/mL LPS for 8 h. Cells were then spun down for 5 min at 400 x g (Sorvall RT6000) and total RNA isolated using Trizol. Briefly, Trizol (1 mL) was added to the cell pellet, and the cells were incubated for 5 min at RT. 200 µL of chloroform (Sigma) was added and the sample shaken vigorously by hand for 15 sec. After a 3-min incubation, the sample was clarified by centrifugation for 15 min at 12,000 x g at 4 $^0$C. The RNA was

subsequently precipitated with 0.5 mL of isopropanol for 10 min at RT, and then centrifuged for 10 min at 12,000 x g at 4 $^0$C. The RNA pellet was next washed twice with 75% ethanol, and centrifuged again for 5 min at 7,500 x g at 4 $^0$C after each wash. It was then air dried for 7-10 min, dissolved in TE and quantified spectrophotometrically at 260 nm. Integrity and purity of RNA were assessed by both $A_{260/280}$ ratio and formaldehyde gel electrophoresis. RNA used in RT-PCR experiments generally had $A_{260/280}$ ratio of greater than 1.8. RNA samples were immediately used for reverse transcription reactions. Remaining RNA was stored at -70$^0$C.

### 2.2.8 RT-PCR

1.5 - 2 µg of total RNA was used to prepare cDNA in a 20 µL reaction volume. Briefly, to the RNA sample 25 µg/mL Oligo $(dT)_{12-18}$ and 0.5 mM dNTP (all reagents for reverse transcription reactions were from Invitrogen Life Technologies) were added. The mixture was heated for 5 min at 65 $^0$C and chilled on ice for a few minutes. After a quick spin (15 sec), a mixture containing 1 x first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 5 mM $MgCl_2$),10 mM dithiotreitol (DTT), and 40 U RNaseOut Recombinant Ribonuclease Inhibitor was added to the sample, which was incubated for 2 min at 37 $^0$C. 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase was then added to the mixture, and the sample further

incubated for 50 min at 37 $^0$C. The reaction was then heat inactivated for 15 min at 70 $^0$C. The resulting cDNA was used as a template for amplification by PCR. In some experiments, reverse transcription with $^{32}$P incorporation was done to ensure validity of the reverse transcription.

For amplification of NOS II and housekeeping GAPDH genes, 2 µL of the cDNA was resuspended in a PCR mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 2.5 U Taq DNA Polymerase (Invitrogen Life Technologies), and 0.5 µM of gene-specific primers. The final PCR reaction volume was 50 µL.

The primers for PKC-η were AACGAGGAGTTTTGCGCTAA (sense) and TGG TAAAATGTTTGAAGATCCG (antisense) (Oshevskie *et al.*, 1999). The NOS II primers were CGGTTCTACTCCATCAGCTC (sense) and TGCCAGAAACTG CGGAAGGG (antisense). The nucleotide sequence of GAPDH is identical in both human and mouse (National Center for Biotechnology Information). The GAPDH primers used were TCACCAGGGCTGCTTTTAAC (sense) and GGAGGC ATTGCTGATGATCT (antisense). For both NOS II and GAPDH, the cDNA was subject to 30 cycles of 1 min denaturation at 94 $^0$C, 1 min annealing at 60 $^0$C and 30 sec elongation at 72 $^0$C. Cycling parameters for PKC-η were 1-min denaturation at 94 $^0$C, 2 min annealing at 58 $^0$C and 1 min elongation at 72 $^0$C for 35 cycles. The first 5-min denaturation at 95 $^0$C and the final 10-min extension at 72 $^0$C were applied in all three cases. Amplification produced a 264-base pair (bp) fragment for

the NOS II gene, a 400-bp fragment for GAPDH, and a 259-bp product for PKC-η. A mock reaction, which contained all the PCR reagents but no template, was prepared in parallel to eliminate false positive amplification. Samples which had all reagents minus the reverse transcriptase served as another negative control. Nine µL of PCR products was gently mixed with 2 µL of 6 x loading buffer (0.25% bromophenol blue [BPB], 4% sucrose, 0.25% xylene glycol FF). The sample was then loaded on a 1.0-1.5% agarose gel containing 0.5 µg/mL ethidium bromide (EtBr), and electrophoresed in a mini-sub cell GT electrophoresis cell (Bio-Rad) at constant voltage (100 V) for 35 min. DNA was visualized under a UV light.

### 2.2.9 SOUTHERN BLOT HYBRIDIZATION

The following procedure was performed at RT, unless stated otherwise. Following electrophoresis, the gel was incubated in denaturing buffer (0.5 M NaOH, 1.5 M NaCl, pH 8.3) for 30 min, followed by two 45-min incubation periods in neutralizing buffer (1.5 M NaCl, 1 M Tris-HCl, pH 7.4). A gel sandwich was assembled, and DNA transferred by gravity capillary transfer to a nylon membrane (HyBond-N, Pharmacia) for overnight. The blot was then baked for 2 h at 80 $^{0}$C, prehybridized for 1 h at 65 $^{0}$C in a 6 mL solution containing 6 x SSC (500 mM NaCl, 50 mM sodium citrate, pH7.0), 1% SDS, 5 x Denhart's solution, and 100 µg/mL denatured salmon sperm DNA, and probed for overnight at 65 $^{0}$C with a $^{32}$P-labelled

recombinant NOS II fragment ($10^6$ cpm). The blot was then washed twice in 10 mL (each time) of 2 x SSC/0.1%SDS at RT, followed by two additional washes of 0.1 x SSC/0.05%SDS at 65 $^0$C. The blot was then briefly dried, sealed with one layer of Saran wrap, and exposed by radiography for 24 h at -80 $^0$C.

## 2.2.10 PREPARATION OF CELL EXTRACTS

### 2.2.10.1 For assessment of PKC protein expression

Monolayers or cell suspension were washed twice with ice-cold PBS, scraped (with a rubber policeman for monolayers), and centrifuged for 5 min at 200 x g (Sorvall RT6000). The cell pellet was resuspended in 100 µL (per 4 x $10^6$ cells) of a cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM ethylene diamine-tetraacetic acid (EDTA), 0.5 mM ethylene glycol-bis-(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), 86 µg/mL phenylmethyl-sulfonyl fluoride (PMSF) and 10 µg/mL aprotinin, and left on ice for 30 min with two 10-sec vortexing periods. The extract was next passed repeatedly through a 26.5 G syringe, and left on ice for another 30 min with two 10-sec vortexing periods. The homogenate was then clarified by centrifugation for 12 min at 11,000 x g (Eppendorf) and the supernatant collected. Protein levels were quantified using a protein assay kit (P 5656) purchased from Sigma. Samples were subsequently stored at -70 $^0$C until used.

### 2.2.10.2 For assessment of NOS II protein expression

After J774A.1 cells were treated either with LPS alone or LPS in the presence of TwHf compounds, 220 µL of boiling lysis buffer (125 mM Tris, 5% glycerol, 4% SDS, pH 6.8) was added to each well of a 6-well plate. The cells were gently scraped with a disposable cell scraper. Viscosity was reduced by pipetting the sample through a pipette tip several times. The cell homogenate was transferred to a 1.7-mL Eppendorf tube, and boiled for 5 min. Subsequently the sample was clarified after 5 min centrifugation at 13, 000 x g at 4 $^{0}$C in a microfuge. Protein levels were measured as described, and samples stored at -70 $^{0}$C until used (no later than 2 days).

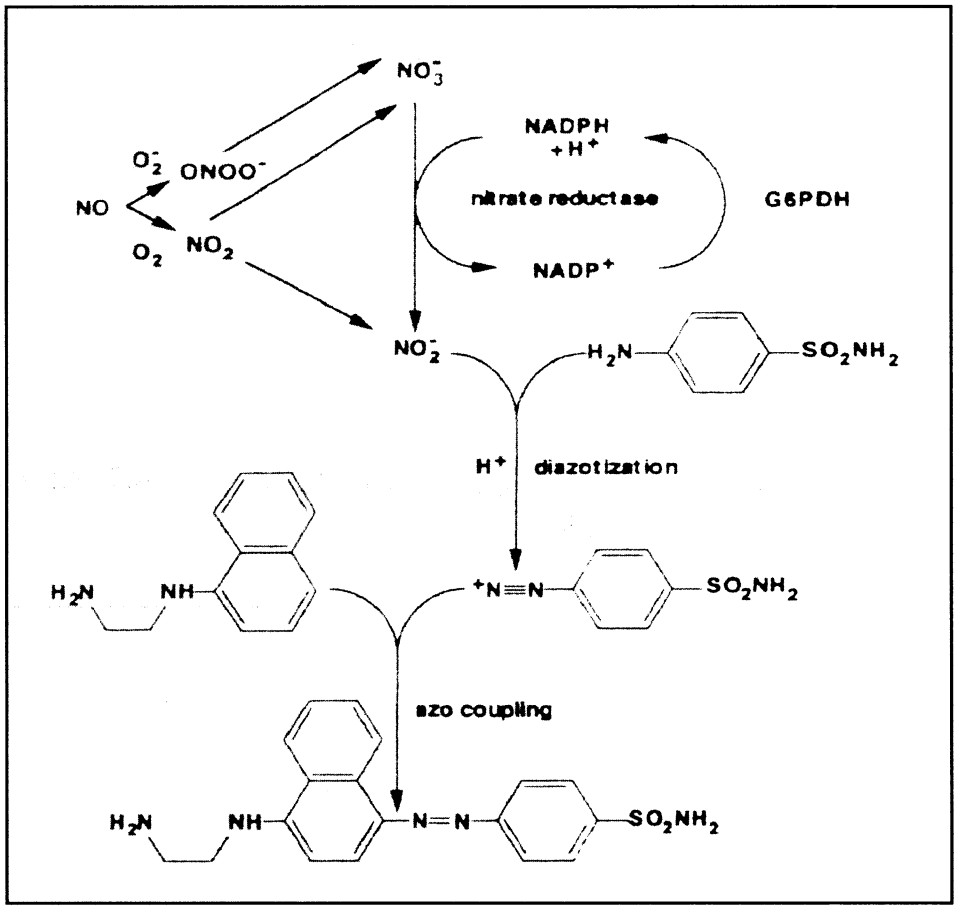
### 2.2.11 WESTERN BLOTTING

Cell lysates (110 µg) were mixed with an equal volume of Laemmli sample buffer (200 mM Tris -HCl, pH 6.8, 20% glycerol, 4% SDS, 1 mM DTT), boiled for 5 min, and resolved on a 10% polyacrylamide gel by electrophoresis for 1 h at constant voltage (100 V) in SDS running buffer (25 mM Tris, 250 mM Glycine, 1% SDS). Molecular weight of samples was determined from the bench-mark protein ladder (Invitrogen Life Technologies). At completion, the gel was washed in cold Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) three times (5 min each time) to remove the SDS, and transferred to a nitrocellulose

membrane (Gelman Sciences, MI, USA) for 5 h at RT (with cooling to reduce heat generation). Efficiency of the transfer was checked by subsequently staining the gel for 30 min at RT in 0.05% Coomassie Brilliant Blue (CBB) solution (10% acetic acid, 25% isopropanol). After blotting, the membrane was successively blocked with 5% non-fat dried milk in Tris-buffer saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) overnight at 4 $^{0}$C, probed with rabbit polyclonal (or mouse monoclonal) anti-PKC or anti-NOS II antibodies for 4 h at RT, and re-incubated for 1 h at RT with appropriate (anti-rabbit or anti-mouse) horseradish peroxidase-conjugated secondary antibodies. Antibody solutions were prepared in low salt TTBS (TBS plus 0.2% Tween-20) containing 0.05% non-fat dried milk. The membrane was washed three times (15 min each wash) with high salt Tween-Tris buffer saline (TTBS; 20 mM Tris, 0.5 M NaCl, 0.2% Tween-20, pH 7.6) between the two antibody incubation periods. In some experiments, specificity of the primary antibody binding was assured by pre-incubating, as per protocol, the primary antibody with excessive amounts (5 times by weight) of an appropriate competing peptide overnight at 4 $^{0}$C before use.

The peroxidase activity was detected with Amersham's ECL detecting kit (Cat #: RPN 2108) and Hyperfilm$^{TM}$ -ECL high performance chemiluminescence film from Amersham. Briefly, a developing solution prepared from addition of 800 µL each of the provided reagents 1 and 2 was overlayered over the whole surface area of the blot, which was then left undisturbed for 1 min in the dark. Radiographs were

**Figure 2.1: Reaction mechanism underlying nitrite determination by the Griess assay**




Taken from Feelisch et al., 1996, p. 494

photographed, digitized, and analyzed by densitometry using a Chemi Imager (4000 Imaging system: Alpha Innotech Corp., San Leandro, CA, USA).

### 2.2.12 DETERMINATION OF NITRIC OXIDE LEVELS

#### 2.2.12.1 Detection in cell culture media

NO is an extremely active free radical with the half-life of about 10-15 sec. In the presence of molecular oxygen, the free radical is spontaneously converted to nitrite, a much more stable derivative, in a 1:1 ratio. In cell culture most, if not all, NO is present in the nitrite form. For this reason, we assessed NO production by indirectly measuring the levels of nitrite by the Griess assay (Fig. 2.1). Unless otherwise stated, all reagents were obtained from Sigma. In brief, 100 µL of cell-free supernatants was well mixed with 100 µL of the Griess reagent (50 µL of 1% (w/v) sulfanilamide in 5% $H_3PO_4$ plus 50 µL of 0.1% (w/v) N-(1-naphthyl) ethylene diamine in water), incubated in the dark for 15 min, and measured for optical density at 590 nm using a microplate reader (Bio-Rad) It should be noted that although not highly sensitive, the measurement of nitrites by the Griess method provides an adequate assessment of NOS II expression because the continuous production of NO by the enzyme, combined with the demonstrated stability of nitrites results in sufficiently high nitrite concentrations.

For measurements of nitrite levels which were below the limits of detection of the Griess assay (2 µM), nitrite measurements were done using a fluorometric

assay which is 50-100 times more sensitive than the Griess assay (Misko, 1993). The fluorometric method basically makes use of the fact that non-fluorescent 2,3-diaminonaphthalene (2,3-DAN) reacts with nitrite in acidic conditions and generates a fluorescent product 1(*H*)-napthotriazole in a 1:1 ratio. As a result, the fluorescence intensity directly correlates with the amounts of nitrite present. Briefly, 10 µL of freshly prepared (0.05 mg/mL in 0.62 M HCl) was added to 100 µL of cell-free supernatants, and mixed immediately. After 10 min-incubation in the dark at RT, the reaction was terminated with 5 µL of 2.8 N NaOH. The intensity of the fluorescence signal was determined using a double beam fluorescence spectrophotometer (Perkin-Elmer 512) with excitation at 365 nm and emission at 450 nm. A series of solutions with known concentrations of purified sodium nitrite (Sigma) prepared in the same media was used to construct a standard curve, from which the unknowns were to be extrapolated. Samples which lacked either sulfanilamide or N-(1-naphthyl)ethylenediamine of the Griess reagent were used as negative controls.

### 2.2.12.2 Detection in biological fluids

Unlike in cell culture where NO is mostly present in the nitrite form, nitrate is the more predominant derivative of NO in plasma. Therefore, in order to quantify NO levels in the circulation, it is necessary to convert nitrate to nitrite first, and then measure nitrite by the Griess assay, as described in Section 2.2.12.1. Briefly,

plasma samples (50 µL) from patients or healthy individuals were diluted four fold with sterile distilled water. 90 U/L *E.coli* nitrate reductase and 44 µM NADPH/FAD (Boehringer, Mannheim, USA) were added to the diluted plasma and the samples incubated for 24 h at RT. Excess NADPH in the samples was eliminated with a mixture containing 480 U/L of L-glutamic dehydrogenase, 4 mM α-ketoglutaric acid, and 84 mM $(NH_4)_2SO_4$ (Sigma). The samples were further incubated for 20 min at RT, deproteinated with 15 g/L $ZnSO_4$, vortexed and clarified by centrifugation for 5 min at 10,000 x g. Total levels of nitrite and nitrate were determined by the Griess assay, as described above (Section 2.2.12.1).

### 2.2.13 ASSESSMENT OF TNF-α PRODUCTION BY L929 FIBROBLAST CYTOTOXICITY BIOASSAY

This bioassay is based on the principle that fibroblast L929 cells are sensitive to TNF-α in the presence of actinomycin D (1 µg/mL, [Merck Sharpe & Dohme International, Rahway, NJ, USA] ). Cells were normally seeded in a 75-$cm^2$ flask at 5 x $10^4$/mL, and cultured for 3-4 days (with media changed, if necessary) until they reached confluency. Confluent cells were successively trypsinized with 25 mL EDTA-Tris solution for 15 min at 37 $^0$C, washed twice with PBS, and once with medium. The cells were next plated in a 96-well plate at a density of 3 x $10^5$/mL (75 µL each well) for overnight. Equal confluency was ensured to allow for assay reproducibility. 75 µL (per well) of samples, containing cell-free supernatants

from test samples or dilutions of purified TNF-α, were added. The latter were used for enumeration of TNF-α levels in the unknowns. After the overnight incubation, the medium was removed and the cells were fixed in 5% paraformaldehyde (PFA)/PBS for 15 min at RT. The plate was washed gently under running tap water until PFA was completely removed. Subsequently, the cells were stained with 1% of aqueous crystal violet dye (prepared in distilled water) for 15 min at RT. Excess dye was removed with running tap water. The plate was left air-dried for 15 min at RT. 100 µL of 33% of aqueous glacial acetic acid was added to each well, and optical density was read at 405 nm on microplate reader (Bio-Rad).

### 2.2.14 ZYMOGRAPHY

This technique was employed to investigate the induction of MMPs. Zymography involves the electrophoretic separation of proteins under denaturing (with SDS) but non-reducing (no DTT or 2-ME) conditions through a polyacrylamide gel containing gelatin, which serves as an *in situ* substrate for MMPs (Hawkes *et al.*, 2001). In brief, cell-free cultures were mixed with equal volume of 2 x sample buffer (125 mM Tris, 50% glycerol, 8% SDS, 2% BPB, pH 6.8), heated for 3-5 min at 55 $^{0}$C, cooled for 2 min, and resolved on a 10% gelatin gel (Bio-Rad, Cat #: 161-1185) by electrophoresis for 2 h (with cooling) at constant voltage (30 V) in SDS running buffer (247 mM Tris, 1.91 M glycine, pH 8.3). Purified trypsin was run along side as a positive control. Upon completion, the gel was treated as follows, on a

shaker at 37 $^0$C, unless otherwise stated. The resolved proteins were renaturated twice for 15 min each time in 100 mL of 2.5% Triton X-100 (Sigma). Subsequently, the gel was incubated in 100 mL of a development buffer (50 mM Tris, 200 mM NaCl, 5 mM anhydrous $CaCl_2$, 0.02%, Brij-35, pH 7.5) for overnight, and then stained in a solution containing 0.5% CBB, 10% acetic acid, and 40% methanol for 1 h at RT. The gel was de-stained with 10% acetic acid until clear bands were observed (representing proteolytic activity of the studied MMPs) against a blue background of the undigested gelatin. Band density was quantified by densitometry using a Chemi Imager (Alpha Innotech Corp.).

### 2.2.15 DATA AND STATISTICAL ANALYSIS

Data were analyzed by software Graph Pad Prism (San Diego, CA, USA) and the results were expressed as mean ± SEM, unless otherwise stated. Where appropriate, statistical analyses (unpaired two-tailed Student's *t*-tests) were carried out using the INSTAT software (San Diego, CA, USA). Probability values of less than 0.05 were considered statistically significant.

# CHAPTER THREE

# INVESTIGATION OF NITRIC OXIDE PRODUCTION AND INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN MONOCYTIC CELLS FOLLOWING CYTOKINE AND LPS STIMULATION

## 3.1 INTRODUCTION

It has been shown that murine monocytic cells exposed to LPS expressed NOS II leading to the production of high amounts of NO (Dimmeler *et al.*, 1997; Chang *et al.*, 1992). For many years, similar experiments have been attempted in human monocytic cells, but the results have been essentially conflicting. However, most agree that LPS- treated human cells do not appear able to release NO *in vitro* at levels comparable to their murine counterparts (Weinberg, 1998; Zemabala *et al.*, 1994). When infected with microorganisms like *Leishmania infantum* (Panaro *et al.*, 1999) and *Mycobacterium tuberculosis* (Chang *et al.*, 1992), or exposed to HIV protein gp120 (Pietraforte *et al.*, 1994), or stimulated with tumor cells (Zemabala *et al.*, 1994), human monocytic cells were able to make NO at measurable levels, albeit at remarkably lower quantities than those observed in their murine counterparts. Human macrophages collected from the lungs of patients with tuberculosis have also been shown to express NOS II (Nicholson *et al.*, 1996).

To this date, the underlying biochemical explanation for the difference is still not known. However, several theories have been proposed to explain the phenomenon. For instance, it was suggested by Tzeng and colleagues (1995) that deficiency of biopterin, one of the co-factors required by NOS II could result in a non-functional NOS II. It was also proposed that the failure of human monocytic cells to produce NO could be due to the absence of the NOS II enzyme, as

introducing the gene into cells, which do not express otherwise, resulted in expression of the NOS II mRNA/protein following LPS stimulation (Bertholet and Manuel, 2000). Yet, some believed that human monocytic cells do not synthesize NO simply because the evolution is such that NO may not be as important to the human immune system, as it is to that of less advanced species (Denis, 1994). However, it would be prudent to re-examine this line of thought as there is increasing evidence for a link between the over-expression of NOS II and pathogenesis of various chronic inflammatory conditions including RA, multiple sclerosis (MS), IDDM, and AIDS/HIV-associated dementia (Chapter 7 for references). A closer look at a role that NOS II and NO may play in inflammatory disease processes is found in Chapter 7.

### 3.1.1 Rationale and aims

The main objectives of the investigation presented in this chapter were to re-assess the induction of NO by both human and murine monocytic cells in response to diverse stimuli including LPS, PMA, and inflammatory cytokines. This was to be done using several murine and human monocytic cell lines, as well as MDM generated from healthy human volunteers.

Partial data reported in this chapter has been published in the journal *Nitric oxide: Biology and Chemistry* (Pham *et al.*, 2003a).

## 3.2 MATERIALS AND METHOD

### 3.2.1 Materials

Stock concentrations of LPS were prepared in PBS to a final concentration of 1 mg/mL, and stored at -20$^0$C until used. PMA was dissolved in reagent grade absolute ethanol and dispensed in 20 µg/mL aliquots, which were then evaporated to dryness. The PMA samples were stored desiccated at -20$^0$C, and resuspended in PBS as needed.

### 3.2.2 Cell stimulation with LPS and cytokines

Cells were grown to sub-confluence. Murine Raw 264.7 and J774A.1 were plated at 1.5 x $10^5$ cells per well in 96-well plates. After 3 h in culture, cells were spun down and fresh media containing appropriate concentrations of LPS was added to the cells. For stimulation experiments with the human cell lines (MM6, THP-1, and U937), trial studies were performed with different numbers of cells (1.5 x $10^5$ - 8 x $10^5$ per well) and at different time points (24, 48, 96, 120, and 144 h). In the end, we found that after 48 h of stimulation, cell viability began to drop significantly, and that there was virtually no change in the cell number up to that point. Therefore, in subsequent studies, murine monocytic cells were treated with LPS for 24 h and human cells were exposed to the bacterial endotoxin for 48 h.

The human monocytic cells were plated at $8.0 \times 10^5$ cells/well in a 24-well petri dish and cultured for 3 h. LPS (or PMA) solutions were subsequently added, and the cells were treated for indicated length of time. Cell-free supernatant was then analyzed for nitrite, as described (Section 2.2.16.1).

In certain experiments where synergistic effects of IFN-γ and TNF-α on LPS-induced NO production by monocytic cells were investigated, the following protocol was adopted. Cells were treated with IFN-γ (50 μg/mL) and TNF-α (0.5 pg/mL), individually or in combination, for 24 h, and then with LPS (0.25 μg/mL) for another 24 h (murine) or 48 h (human). No such co-stimulation was done for PMA and the cytokines. Cell-free supernatants were then analyzed for nitrite, as above. A sample containing only medium, treated in parallel, was also measured for nitrite. This served as a baseline value which would be taken into consideration in data analysis.

### 3.2.3 Analysis for display of NOS II protein and expression of mRNA following LPS stimulation

Murine or human monocytic cell lines ($4 \times 10^6$ cells/100-mm petri dish) were stimulated with 1 μg/mL LPS for 13 h. Total cell extracts were prepared, as described (Section 2.2.10.2) and the samples were screened for NOS II protein by western blotting (Section 2.2.11).

To evaluate the effect of LPS on transcription activity of NOS II in monocytic

cells, the following experiment was conducted. MM6 and J774A.1 cells ($10^6$ per dish) were treated with 1 µg/mL LPS for 8 h. Total RNA were extracted (Section 2.2.7), and subjected to RT-PCR analysis (Section 2.2.8) with NOS II-specific primers. Specificity of positive signals on the agarose gel was confirmed by Southern hybridization (Section 2.2.9) using a $^{32}$P-labelled recombinant NOS II fragment.

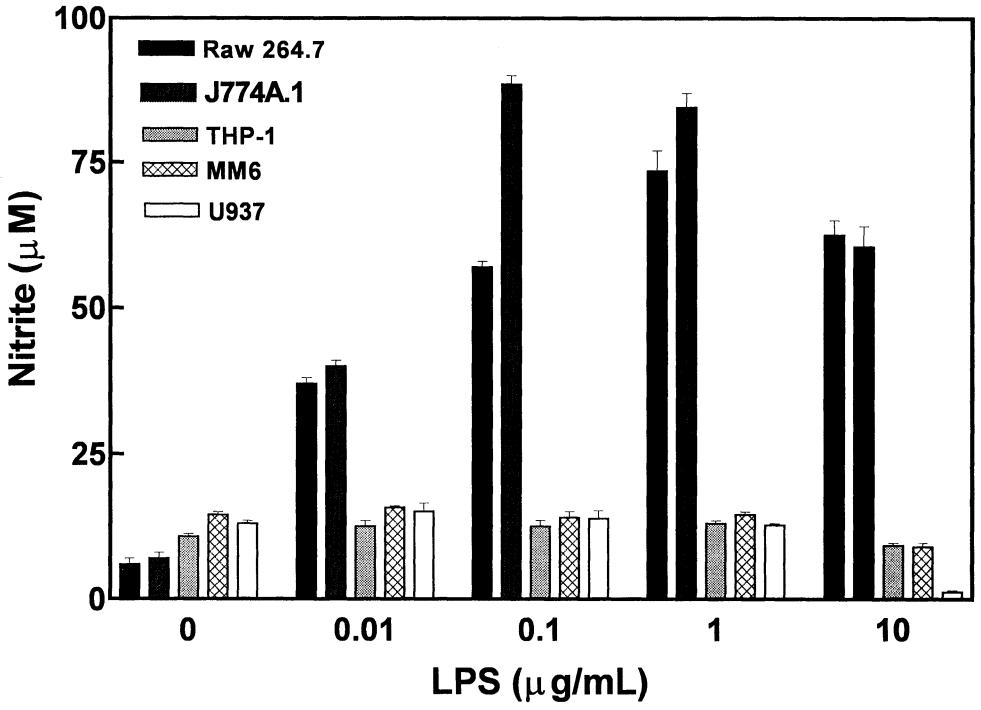
## 3.3 RESULTS

### 3.3.1 NO production following LPS, cytokine and PMA activation

Monocytic cells from murine Raw 264.7 and J774A.1 and human U937, MM6, and THP-1 cell lines were stimulated with increasing concentrations of LPS (10 ng/mL-10 µg/mL) for 24 h (murine) and 48 h (human). Cell-free supernatants were subsequently analyzed for nitrite. To obtain a more accurate measurement of low NO levels (less than 2 µM), we chose the fluorometric DAN assay, which is 50-100 times more sensitive than the popular colorimetric Griess assay. Precautions were made to ensure that there were no significant inherent variations between the two assays. It was clear from the data (Fig 3.1) that there was a marked dose dependent increase in NO production ($p < 0.001$) by LPS-stimulated monocytic cells from the two murine cell lines Raw 264.7 and J744A.1. In contrast, cells from the three human cell lines tested MM6, U937, and THP-1 failed to

**Figure 3.1: LPS-induced nitric oxide production in human and murine monocytic cells**

Murine Raw 264.7 and J774A.1 ($1.5 \times 10^5$ per well) or human THP-1, MM6, and U937 ($8 \times 10^5$ per well) monocytic cells were stimulated with various concentrations of LPS for 24 h (murine) or 48 h (human). Cell-free supernatant was measured for nitrite colorimetrically by the Griess assay or fluorometrically with 2,3-DAN, as detailed in Chapter 2 (Section 2.2.12.1). Sodium nitrite was used as a standard to construct a standard curve from which the unknowns were extrapolated. Results are expressed as mean (± SD, n=3, quadruplicate experiments) NO induction by $10^6$ cells in 24 h after subtraction of blank (sample contained only media).


100
75
50
25
0
Nitrite (μM)
Raw 264.7
J774A.1
THP-1
MM6
U937
0
0.01
0.1
1
10
LPS (μg/mL)

produce any detectable amounts of NO. Different experimental conditions including higher cell number and longer incubation periods were also tested in an effort to induce a response in these human cells. Still, the results remained unchanged. When murine J774A.1 monocytic cells were primed with a cocktail of IFN-γ and TNF-α for 24 h prior to LPS stimulation, the response was further enhanced ($p<0.001$), as shown by augmentation of two-fold in NO production relative to that of LPS alone (Fig. 3.2). Co-stimulation of either IFN-γ or TNF-α with LPS did not lead to any upregulation in nitrite levels suggesting that the two cytokines complement each other in delivering a "signalling boost". In contrast, human monocytic cells from the MM6 cell line remained essentially unresponsive regardless of the type of treatment.

Murine cells J774A.1 stimulated with PMA showed a notable increase in NO released by the murine cells into the cell culture, albeit the response was significantly weaker than that induced by LPS (Fig. 3.3). Similar investigation with human cells MM6 yielded no induction of NO following their activation with PMA.

### 3.3.2 Effects of LPS on the induction of NOS II protein in human and murine monocytic cells

Our data reported here, as well as those of others, have provided strong and consistent evidence for the lack of LPS and/or cytokine-mediated

**Figure 3.2: LPS and cytokine co-stimulation further enhances NO production by murine monocytic cells.**

Murine J774A.1 (1.5 x $10^5$ per well) and human MM6 (8 x $10^5$ per well) cells were primed with IFN-γ (50 µg/mL) and TNF-α (0.5 pg/mL), individually or in combination for 24 h. The cells were next exposed to 0.25 µg/mL LPS for 24 h. Cell-free supernatants were measured for nitrite, as described (Section 2.2.12.1). The results were expressed as the mean value (of NO produced by $10^6$ cells in 24 h) ± SD of three quadruplet experiments. Statistical significance of the data was evaluated by unpaired Student's *t*-tests.

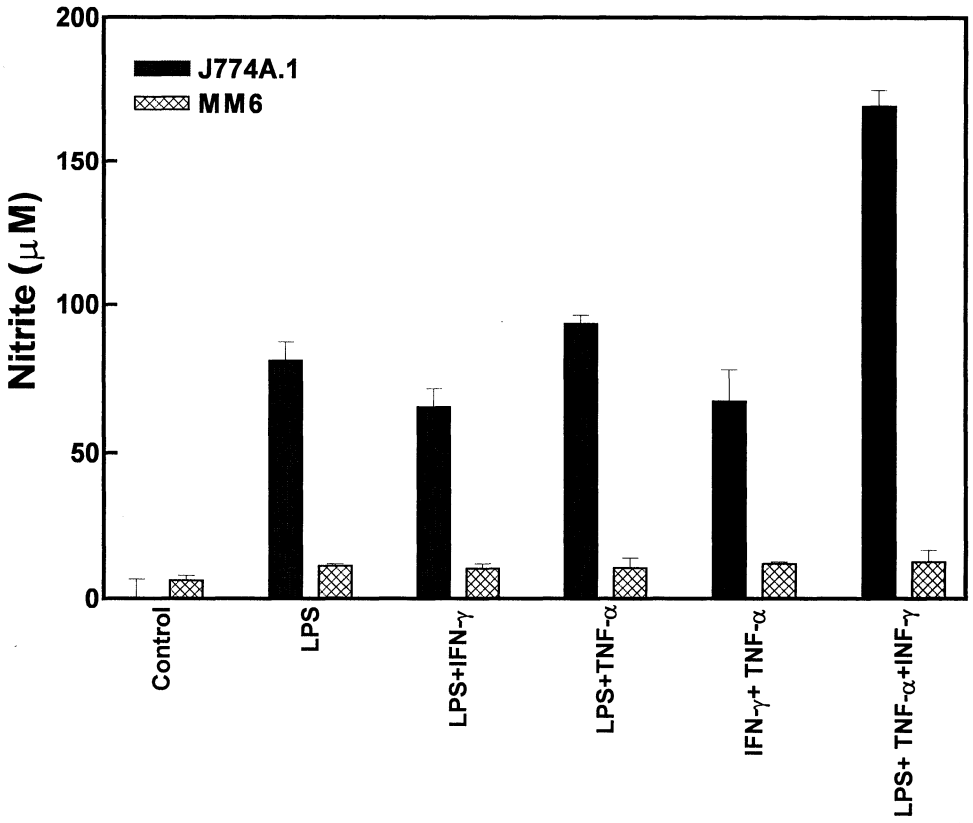
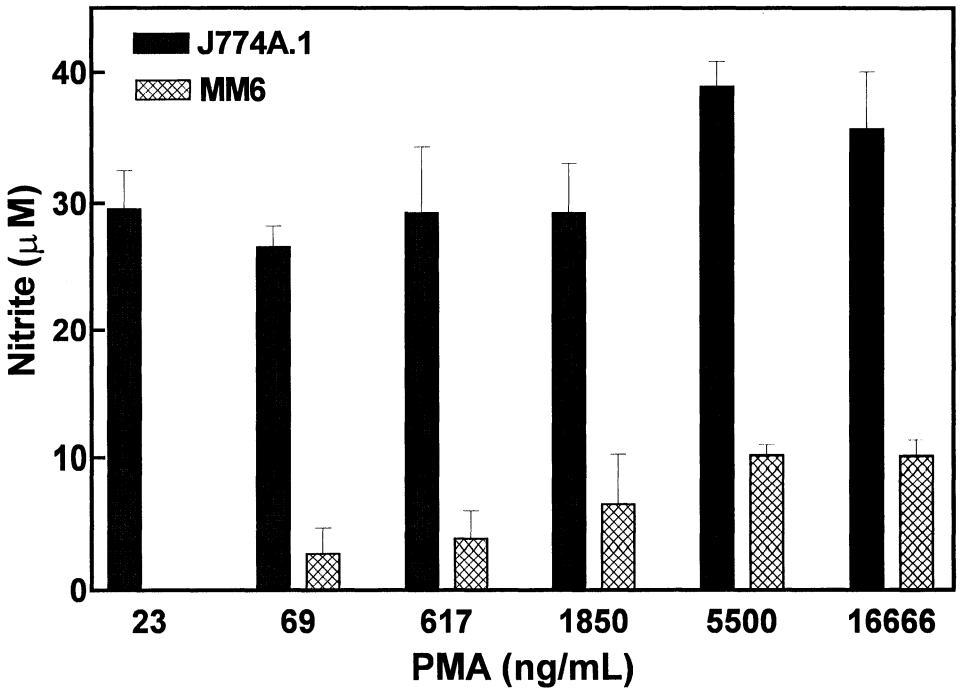

200
150
100
50
0
Nitrite (μM)
J774A.1
MM6
Control
LPS
LPS+IFN-γ
LPS+TNF-α
IFN-γ+ TNF-α
LPS+ TNF-α+INF-γ

**Figure 3.3: Effects of PMA on NO production by murine monocytic cells**

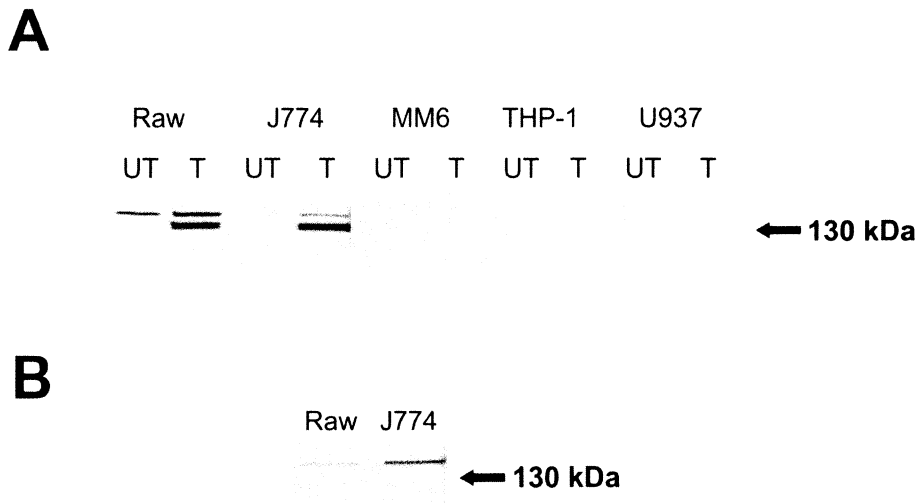
Murine J774A.1 cells ($1.5 \times 10^5$ per well) was plated in each well of a 96-well plate, and cultured for 7 h. After which time, old media was removed and fresh one containing appropriate doses of PMA. The cells were incubated for another 24 h, and cell-free supernatants were harvested for nitrite measurements, as described. The results were expressed as mean (of NO produced by $10^6$ cells in 24 h) ± SD of two quadruplicate experiments, after the background (if any, released by untreated cells) had been subtracted.


J774A.1
MM6
Nitrite (μM)
0
10
20
30
40
23
69
617
1850
5500
16666
PMA (ng/mL)

NO synthesis in human monocytic cells, at least at the levels to be accurately measured in culture, when compared to their murine counterparts (Weinberg *et al.*, 1995; Zemabala *et al.*,1994; St-Denis *et al.*, 1998). This raises an interesting question as to whether the differences in NO-generating potential might be attributed to either the absence of the NOS II enzyme itself or insufficient, or lack thereof of, *de novo* quantities of the co-factors. In an attempt to answer the question, we investigated the induction of NOS II by monocytes in response to LPS using western immunoblotting. As evident in Figure 3.4 A, there was evident induction of NOS II (arrow, 130 kDa) in both LPS-stimulated Raw 264.7 and J774A.1 cells, but not in any of three human cell lines tested. Binding specificity of the antibody was later confirmed by preincubation with a NOS II blocking peptide (a 20-amino acid fragment mapped to the N-terminus of the protein), which was originally used to raise the antibody in the rabbit (Fig. 3.4 B). As shown, incubation of the antibody with the NOS II peptide prior to testing of the samples resulted in a complete block of the specific band. It should be noted that the presence of the upper band of approximately 178 kDa in the samples is most likely to be due to non-specific binding, as it was not blocked by the competing peptide. Taken together, we conclude that LPS indeed mediates the induction of the functional NOS II protein in murine, but not in human monocytic cells.

**Figure 3.4: Investigation of NOS II induction by endotoxin in human and murine monocytic cells**

Murine (J774A.1 and Raw 264.7) and human (THP-1, U937, and MM6) monocytic cells ($2 \times 10^6$ cells per 35 mm dish) were stimulated (T) with1 µg/mL LPS for 13 h, and extracted, as described (Section 2.2.10.2). 110 µg of the total cell lysate was tested for NOS II by western blotting (Section 2.2.11) using a rabbit polyclonal anti-NOS II antibody, which recognized the same epitope in human and murine. (**A**), For each cell line, a sample of untreated (UT) cells was prepared in parallel and analyzed under the same conditions as the LPS-treated sample. (**B**), To confirm binding specificity, murine LPS-treated cells were probed with the anti-NOS II antibody, which had been pre-incubated overnight with excess blocking peptide. The positive signal showed the expected size of NOS II (130 kDa), which is not present after pretreatment of the antibody with the blocking peptide.


A
Raw
J774
MM6
THP-1
U937
UT T UT T UT T UT T UT T
130 kDa
B
Raw J774
130 kDa

### 3.3.3 Effect of LPS on NOS II transcription in human monocytic cells

Our findings from Western Blotting have shown convincingly that stimulating human monocytic cells with LPS does not give rise to expression of the NOS II gene at the protein level. Since NOS II expression is mainly regulated at the transcriptional level, we assessed whether LPS could signal gene transcription by RT-PCR. For this experiment, LPS stimulation of monocytic cells was carried out for 8 h for maximal detection of NOS II mRNA (Reiling *et al.*, 1994). As depicted in Figure 3.5, no mRNA was evident for the two human cell samples stimulated with LPS (MDM and MM6) either before or after LPS stimulation. This is in sharp contrast with what was seen in murine J774A.1 where transcription of NOS II was clearly detected. Validity of the RT-PCR results was later confirmed by Southern hybridization of the PCR products to a $^{32}$P-labelled NOS II recombinant fragment (Fig. 3.5 B).

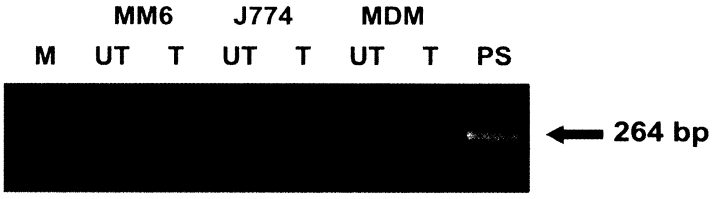
## 3.4 SUMMARY AND DISCUSSION

In this chapter, we examined the ability of human and murine monocytic cells from a series of cell lines as well as MDM (from a healthy volunteer) to produce NO in response to the monocyte-activating agent of choice LPS. We have shown that unlike human monocytic cells, their murine counterparts were able to produce high amounts of NO in response to LPS (Fig. 3.1). While priming the murine cells with

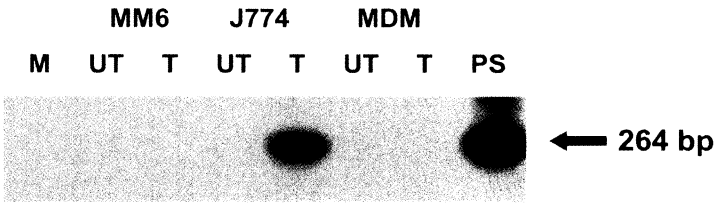
**Figure 3.5: Effects of LPS on the induction of NOS II mRNA in human monocytic cells**

Human (MM6) and murine (J774A.1) monocytic cells ($10^6$ cells per dish) were treated (T) with 1 µg/mL LPS for 8 h. After which time, total RNA was isolated, reverse-transcribed and amplified by PCR using NOS II-specific primers. A sample from untreated (UT) cells was also prepared in parallel and analyzed in the same way as the test sample. A mock (M) contained only water in place of cDNA and a sample contained synthetic NOS II (PS) were used as a negative and a positive control, respectively. (A), Nine µL of PCR products was resolved on a 1.5% agarose gel, which was then digitally photographed and scanned. (B), Detection specificity was confirmed by Southern hybridization of the PCR products from (A) with a recombinant NOS II fragment, as described (Section 2.2.9).

A


MM6
J774
MDM
M
UT
T
UT
T
UT
T
PS
264 bp

B


MM6
J774
MDM
M
UT
T
UT
T
UT
T
PS
264 bp

a cocktail of pro-inflammatory cytokines (IFN-γ and TNF-α) prior to LPS stimulation further augmented nitric oxide synthesis by nearly two-fold, the same treatment did not give rise to any detectable NO that could be measured accurately in cell cultures of human monocytic cells (Fig. 3.2). In the case of murine, further upregulation of NO suggests that these cytokines and LPS act on distinct but related signalling pathways to synergistically induce the transcription of the NOS II gene (Mautino *et al.*, 1994). In addition, the data from Figure 3.2 also showed that the co-stimulation of the murine cells with LPS and individual cytokines did not effect any noticeable changes in the levels of NO produced.

The findings from our western blotting have shown that human monocytic cells treated with LPS did not result in the induction of NOS II protein, suggesting that the inability of these human cells to make NO may be associated with the lack of protein expression. It is not likely that the observations were due to the fact that the NOS II antibody used in our studies did not recognize the human antigen on the basis that the peptide against which the purchased antibody was raised was derived from the highly conserved region shared by both human and murine cells. Thus, our data at this point do not appear to support a hypothesis that the failure of human monocytic cells to release NO following activation by endotoxin and cytokines was due to non-functionality of the NOS II enzymes brought about by the lack or insufficient quantities of the cofactors (e.g. $BH_4$, NADPH, to name a couple), as suggested previously (Bertholet *et al.*, 1999). At the same time, however, we

could not exclude the possibility that the level of NOS II expression in these cells was too weak to be detected by immunoblotting. Thus, we proceeded with examining the presence of mRNA in LPS-activated cells by RT-PCR. At this end, such analyses have shown no evidence of mRNA for NOS II in human monocytic cells stimulated with LPS. In short, our findings as obtained from western blotting and RT-PCR have demonstrated unequivocally that LPS did not trigger transcription of the human NOS II gene supporting the notion that the failure of human monocytic cells to synthesize NO appears to be attributed to gene expression and not its functionality, as suggested previously. In fact, our findings reported up to this point are consistent with studies which showed that once transfected with the NOS II gene, human U937 cells acquire the potential to produce NO in response to LPS, and thereby exhibit leishmanicidal activity (Yan *et al.*, 1997; Bertholet and Manuel, 2000).

As previously discussed in Chapter 1 (Section 1.2.2.2.2), NOS II expression is primarily regulated at the transcriptional level. Our data presented here appears to point towards the direction that there may be some blockage in the signal transduction pathway leading to NOS II induction, and the defect is likely to occur before the transcription. For example, transcription factors involved in the signalling pathway leading to NOS II induction may not be expressed or if they are, may not be functional. There exists also a possibility that the absence of the NOS II gene following activation by LPS could be a result of inherent unresponsiveness of the

cells to the stimulus. This phenomenon could be brought about by a lack of receptor(s) such as CD14 or TLR-4, which are thought to be required for LPS recognition by monocytic cells or due to the lack of key signalling elements in the pathway leading to NOS II induction.

# CHAPTER FOUR

# LPS RECEPTOR EXPRESSION AND LPS-TRIGGERED SIGNALLING PATHWAY IN MONOCYTIC CELLS

## 4.1 INTRODUCTION

### 4.1.1 The role of CD14 and LBP in LPS binding

The cellular signalling events leading to inflammatory responses in monocytic cells activated by LPS have been well studied. Although the complete pathway is yet-to-be fully elucidated, major signalling events have been established.

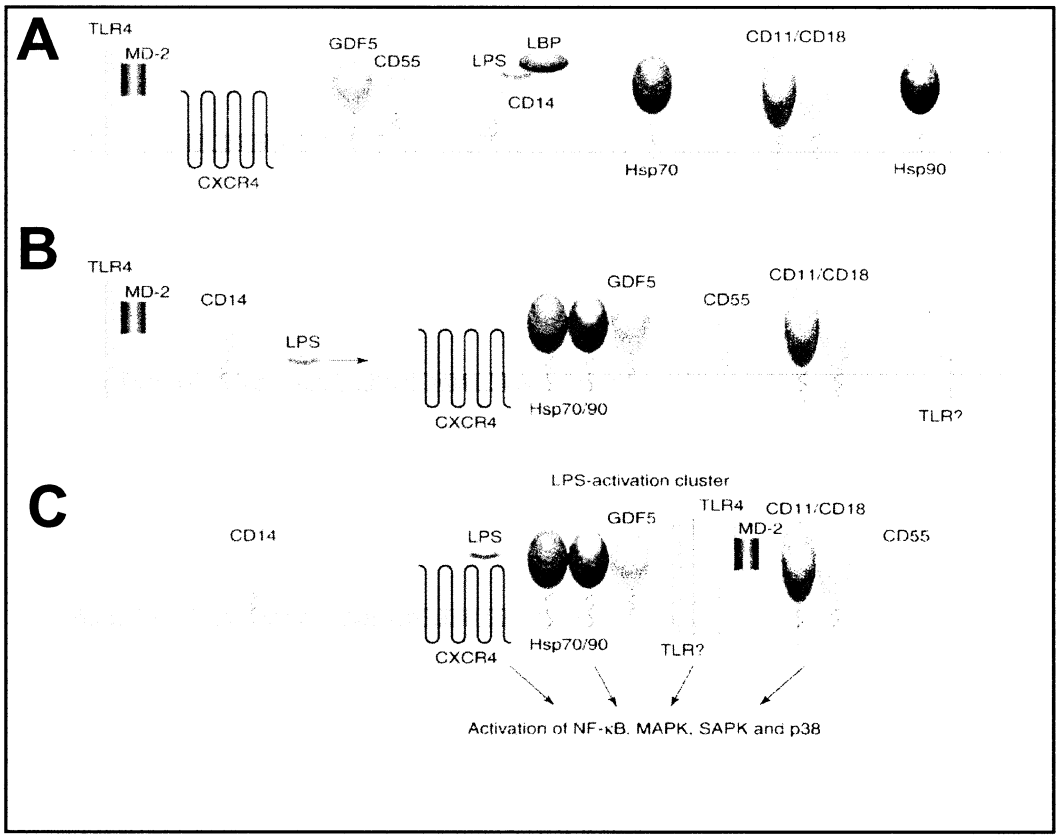
As previously discussed (Section 1.4), LPS is the main ingredient of the outer component of the Gram-negative bacterial cell walls. It is widely accepted that in order for LPS to signal through CD14, the lipid A portion of LPS has to first bind to a 60-kDa serum factor known as LBP. The complex is needed for optimal binding of LPS to the membrane-bound CD14 (Hailman *et al.*, 1994). It is thought that the LBP and lipid A interaction enhances the biological activity of LPS by 100- to 1000-fold. Alternatively, soluble CD14, formed by surface shedding, may also facilitate stabilization of the LPS-LBP interaction. The importance of LBP in LPS recognition has been again demonstrated in a recent study by Fierer *et al.* (2002) where they showed that LBP-knockout mice were more susceptible to Salmonella peritonitis than wild type animals. In this model, these investigators illustrated that the absence of LBP would lead to a defect in chemotaxis of polymorphonuclear phagocytes. The binding of LPS to CD14 on the cell surface is followed by a subsequent cascade of intracellular events leading to phosphorylation and activation of many key signals giving rise to production of inflammatory mediators

including cytokines, RNI, and lipid-derived products (Chow *et al.*, 1995). Over the years, the importance of CD14 in cell responsiveness to LPS has been demonstrated in several studies, including that employing site-directed mutagenesis (Stelter *et al.*, 1997), anti-CD14 antibodies (von Asmuth *et al.*, 1993; Wright *et al.*, 1990), and soluble CD14 (Schutt *et al.*, 1992).

### 4.1.2 The role of TLR-4 in LPS signalling

The fact that CD14 is a glycosylphosphatidyl inositol anchored membrane glycoprotein with no cytoplasmic domain confused scientists for years with respect to the mechanism by which the glycoprotein transduces the signal across the membrane into the cell. As a result, there came forth a hypothesis that there must be other receptor(s) or adapter(s) which could actually transmit the signal. This theory was strengthened by studies which had shown that anti-CD14 antibodies weakened, but did not completely abrogate, LPS signalling, suggesting the existence of other molecules (Blondin *et al.*, 1997; Triantafilou *et al.*, 2000). In addition, it has been observed that C57BL/10ScCr and C3H/HeJ mouse strains, which carry a null allele and a missense mutation at residue 712 (Proline replaced by Histidine) of the *Lps* gene, respectively, do not respond to LPS. After much anticipated work, the *Lps* gene identified by positional cloning was the murine TLR-4, which belongs to the family of PAMPs (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999). Incidentally, the missense P712H mutation was found to be located in the

**Figure 4.1: Proposed LPS-triggered signalling pathway in monocytic cells mediated by CD14 and TLR-4**




Adapted from Triantafilou and Triantafilou (2002) Trends Immunol.: **23**,301-304.

signalling domain of the TLR. TLR4 is the most well characterized isoform of the mammalian homologues of the *Drosophila* Toll receptor first identified as a multi-functional molecule with antifungal properties (Lemaitre *et al.*, 1996). TLR4 is a single-spanning transmembrane protein with a leucine rich ectodomain and a cystoplasmic domain with a Toll/IL-1 like sequence. The importance of TLR4 in LPS signalling has been demonstrated in gene-specific mutations and knock out mice (Triantafilou and Triantafilou, 2002; Hoshino *et al.*, 1999). Specifically, animals that lacked TLR-4 exhibited hyporesponsiveness to LPS and their monocytic cells were not capable of producing inflammatory cytokines, such as TNF-α and IL-1 following LPS activation (Hoshino *et al.*, 1999).

### 4.1.3 LPS-triggered signalling in monocytic cells

By analogy to the immunological synapse, LPS-induced activation of mononuclear phagocytes has been proposed to likely consist of two micro-domains or zones of activation: a periphery and a core region (Triantafilou and Triantafilou, 2002). In this scenario, LPS binds to CD14 via LBP triggering recruitment of other signalling molecules including heat shock proteins (hsp) 70 and 90, CXCR4 and GDF5 (growth differentiation factor 5), and CD55 to the site of LPS/LBP-CD14 ligation. LPS released from CD14 in the lipid bilayer initiates clustering of other signal transducing molecules including the TLR4-MD2 complex interacts with MD2, CD11 and CD18. MD-2 is a secreted protein but found to be associated at the cell

surface with TLR-4 (MD-2 physically interacts with TLR-4 in a similar manner to MD-1 binding to RP-105, a B-cell specific receptor; Miyake *et al.*, 1995). The model proposed by Triantafilou and Triantafilou (2002), CD14 having released LPS may stay in the periphery while other signalling molecules cluster in the core region (Fig. 4.1). The involvement of the receptor cluster in the recognition of LPS explains the multiple signalling cascades triggered by the microbial product. In addition to the extracellular molecules discussed here, the LPS signalling pathway also requires a number of intracellular signalling factors including MyD88, IL-1 receptor associated kinases (IRAK), and TNF-α receptor-associated factor 6 (TRAF-6), to name a few.

LPS has been shown to activate multiple signalling pathways leading to alterations in cell functions. For example, the phospholipase C (PLC)/Calcium/PKC axis is perhaps the most widely studied signal transduction mechanism. The pathway is crucial for the break down of phospholipids by PLC, mobilization of intracellular $Ca^{2+}$ stores, activation of $Ca^{2+}$ channels and PKC. In addition, LPS signalling leads to activation of NF-κB, and mitogen activated protein kinase (MAPK) families, including p38 MAPK, extracellular signal regulated kinase 1 and 2 (ERK1/2), and c-Jun-terminal kinase/stress-activated protein kinases (JNK/SAPK) (Rao, 2001). NF-κB is a very important transcription factor involved in the regulation of many different genes including those coding for pro-inflammatory mediators. The activation of NF-κB first requires phosphorylation and activation of

IκB kinases, namely IKK-α and IKK3 (May and Ghosh, 1999) by upstream kinases (*e.g.,* NF-κB inducing kinase [NIK] and mitogen-activated protein kinase ERK kinase kinase 1 [MEKK-1]). The activated IKK then phosphorylates its substrate, the inhibitory kappa B (IκB) leading to dissociation of IκB from NF-κB, and activation of the latter. Activation of all three MAPK pathways in monocytes by LPS is thought to be mediated by the *src* tyrosine kinase family including $p53/56^{lyn}$ and $p58/p64^{c\text{-}fgr}$ (Sweet and Humme, 1996; Shapira *et al.*, 1994). With regards to the ERK1/2 pathway, it is thought that in monocytic cells, binding of LPS to its receptor complex at the cell surface leads to rapid phosphorylation and then activation of tyrosine kinases (see above). These activated kinases subsequently mediate activation of the MEK-ERK1/2 pathway in a Raf-1-dependent manner (Reimann *et al.*, 1994). This pathway is required for the production of several inflammatory mediators including IL-1, IL-8,TNF-α, and prostaglandin E2 (PGE2). The JNK and p38 pathways are also activated in LPS-activated monocytes and are crucial to the production of several inflammatory mediators including cytokines. Although there may be some degree of functional redundancy between these pathways in terms of the genes they help induce, the different and shared signalling events may contribute to their synergistic effects in regulating cell functions.

In addition to activating PLC, LPS is also involved in the activation of other lipases including the phospholipase A family and phospholipase D (Chow *et al.*, 1995). This group of enzymes is important to the production of lipid-derived second

messengers, such as arachidonic acid.

### 4.1.4 Inflammatory mediators produced by monocytic cells in response to LPS

As mentioned earlier, several transcription factors including NF-κB, c-Jun, STAT, and interferon-responsive factor 1(IRF-1) are activated by LPS in monocytic cells (Nau *et al.*, 2002). These, in turn, are essential to the activation of various genes involved in the defence against microbial pathogens as well as those involved in initiation of adaptive immune response. Some of the inflammatory cytokines known to be activated by LPS are: TNF-α, IL-1β, and IL-6. In addition, the microbial product has also been shown to induce the expression of MMP and of NOS II in monocytic cells. The latter, of course, leads to the production of NO (Chapter 3).

MMPs (matrixins) form a family of highly homozygous zinc proteinases (endopeptidases) divided into four subgroups according to their substrate specificity and structural homology, and degrade at least one component of the extracellular matrix (Bode and Maskos, 2001). Thus, the enzymes allow leukocytes to extravasate across the endothelium into the tissue stroma. In physiological conditions, integrity of the underlying matrix is preserved by the presence of specific tissue inhibitors of metalloproteases (TIMP) (Brew *et al.*, 2000). However,

the balance of MMPs and TIMPs is shifted in certain infections and disease processes such that MMP production is greater while that of TIMP is decreased. A consequence of this, of course, would be structural damage to the underlying tissue.

### 4.1.5 Rationale and aims

The findings described in Chapter 3 indicate that human monocytic cells, both from cell lines and primary cultures, failed to release NO following exposure to LPS. The data also revealed that this is due to the absence of NOS II gene activity on both protein and mRNA levels in the LPS-stimulated cells. In light of the above, it was of interest to determine if the unexpected observation could be attributed to inherent hyporesponsiveness of these human cell lines to LPS. To test this hypothesis, we investigated the expression of TLR4 on MM6 cells, which had not been previously examined. We also wanted to assess the ability of the cells to produce TNF-α, MMP-2, and MMP-9 following LPS treatment to assess whether these cells were responsive to LPS in a known TLR4 signalling-like manner.

## 4.2 METHODS

### 4.2.1 Cell stimulation with LPS for evaluation of TNF-α

Human MM6 cells were seeded in a 24-well plate at 0.6 x $10^6$ cells per well. After cultured for 3 h, the cells were treated with varying concentrations of LPS for

24 h. Cell-free supernatants were evaluated for TNF-α levels by the cytotoxicity bioassay with L929 (Section 2.2.13).

### 4.2.2 Cell treatment for zymography analysis

MM6 cells were washed three times with serum-free (SF) medium (same medium as that used for MM6 and U937 but without FBS, Section 2.1.2.1), plated in a 24-well plate at density $10^6$ cells/mL/well, and cultured for 1 h. In a 1.5-mL centrifuge tube, 50 µL of 10 µg/mL LPS was mixed with 50 µL FBS, and incubated for 30 min at RT to allow for the formation of LPS and serum LPS-binding protein (LBP) complex (Section 4.1.1). The mixture was subsequently serially diluted with the SF medium such that the final LPS concentration in the well was 100 ng/mL. The cells were stimulated for 24 h, and cell-free cultures were assessed for MMP activity by zymography (Section 2.2.14). A sample containing unstimulated cells treated in the same way served as a negative control.

MMP activity was semi-quantified by a ChemiImager (Alpha Innotech Corp.). Densitometry reading, if any, obtained from a sample containing only medium was used as a background. Values for test samples were recorded after the background had been subtracted.

### 4.2.3 Immunofluorescence staining

MM6 cells were harvested, washed once with PBS, and resuspended at $10^6$

cells/mL. The following procedure was done on ice. In a 1.5-mL centrifuge tube, 100 µL of the cell suspension was incubated with 100 µL of 10% of human AB serum (Rh negative) to block Fc receptors. After 1 h, the cells were washed twice with 0.7 mL of a wash/staining buffer (PBS containing 0.05% sodium azide and 1% FBS) for 5 min at 250 x g at RT. The cells were subsequently labelled with 100 µL of mouse APC-conjugated anti-TLR-4 antibody (or an isotype control antibody for a control sample) for 1 h in the dark. The cells were next washed twice (5 min each time) with 1.5 mL wash buffer and spun down for 10 min at 250 x g at RT. Subsequently, they were fixed in 0.5% PFA /PBS for 30 min. After removal of the fixative, the cells were resuspended in 50 µL of the media and mounted on 10% glycerol/PBS. The slide was covered with a size 22 cover-slip. The cells were examined under an Olympus BX50WI (Diagnostics Instruments, Inc.) microscope fitted to a digital camera system controlled by a the SPOT RT software (Version 3.1) operated by a View Sonic Compaq Computer with Windows 95/98/NT.

## 4.3 RESULTS

### 4.3.1 Cell surface expression of TLR4 on human monocytic cells

Cell surface expression of TLR4 was examined by both fluorescence microscopy and FACS. As shown in Figure 4.2, MM6 cells expressed a significant amount of TLR4 molecules. Cells incubated with the secondary antibody in the

absence of the primary antibody showed no evident staining, confirming detection specificity.

### 4.3.2 Production of TNF-α and MMP by human monocytic cells in response to endotoxin stimulation

In light of our findings which showed that human monocytic cells were unable to make NO following LPS stimulation, it was of interest to see if the human cells were able to produce other inflammatory mediators, such as TNF-α and MMP in response to LPS. As shown in Figure 4.3, TNF-α was produced by MM6 upon exposure to LPS, and the response was sharply increased with increasing concentrations, maximized at 1 μg/mL LPS and levelling off quickly at higher concentrations. It was also noted that TNF-α was produced even at levels of LPS as low as 5 ng/mL suggesting the presence of CD14 in MM6, enhancing the effects of the signalling complex.

In addition, we also examined by zymography the expression of two gelatinases MMP-2 and MMP-9 produced by MM6 cells. The principle of this assay is based on the observation that when the gelatinases produced by activated macrophages are resolved by electrophoresis on a gelatin-embedded gel, the enzymes will cleave the gelatin substrate leaving clear areas at the site where gelatin is digested. The band is visible after the gel has been developed and stained with Coomassie blue (Fig. 4.4 A). Figure 4.4 B shows the level of

**Figure 4.2: Cell surface expression of TLR-4 on human MM6**

MM6 ($10^5$ cells in100 µL) cells were incubated with 5% normal human AB serum to block Fc receptors. Subsequently, the cells were stained with APC-conjugated mouse anti-human TLR4 antibody (MM6-TLR4). In a control sample, MM6 cells were stained with an APC-conjugated isotype control antibody (MM6-IgG) instead of the TLR-4 antibody. After fixation, TLR-4 expression was assessed by immunofluorescence.

MM6-IgG

MM6-TLR4

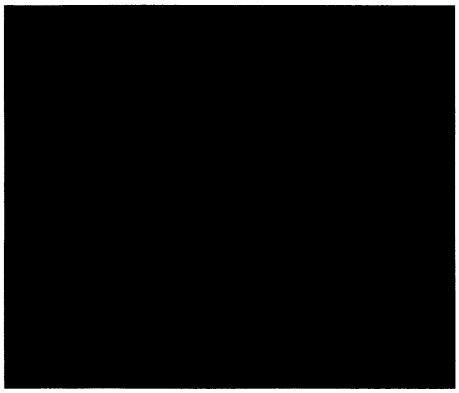
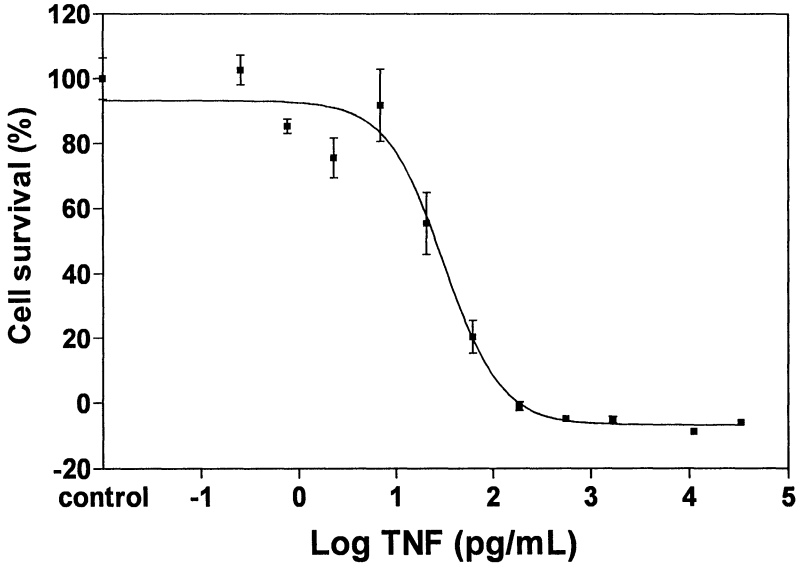


**Figure 4.3: LPS-induced TNF-α production by human monocytic cells**

Human MM6 cells (600 µL of 1 x $10^6$ cells/mL) were exposed to varying concentrations of LPS for 24 h. Cell-free supernatants were collected for the bioassay with L929, as detailed in Section 2.2.13. (**A**), A standard curve was prepared for each bioassay with purified TNF-α to permit translation of cytotoxicity into linear units of TNF-α concentrations. (**B**), Evaluation of TNF-α production of the MM6 cells in response to the stated doses of LPS was determined from the standard curve in (**A**). All measurements were performed in triplicate, and the results were expressed as mean ± SEM.

A


120
100
80
60
40
20
0
-20
Cell survival (%)
control
-1
0
1
2
3
4
5
Log TNF (pg/mL)

B

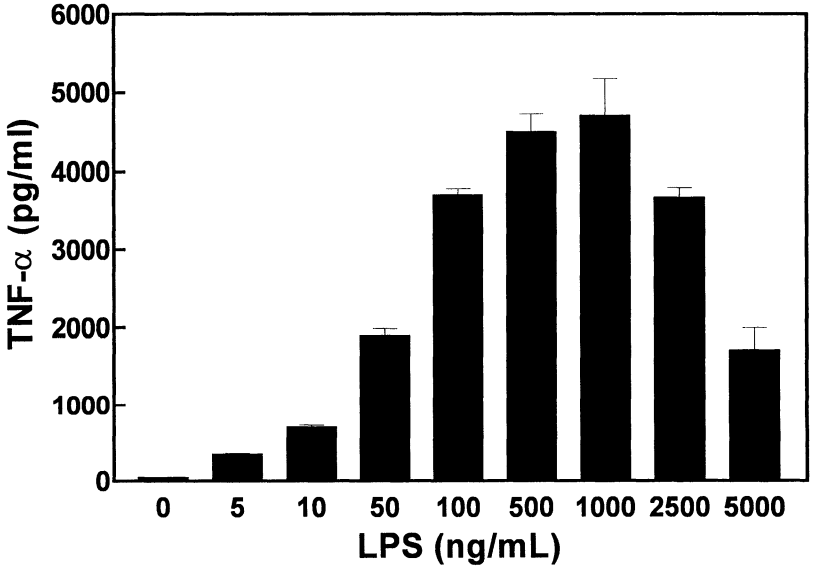
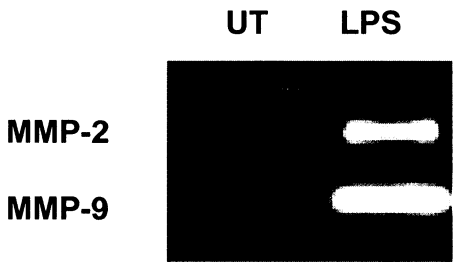

6000
5000
4000
3000
2000
1000
0
TNF-α (pg/ml)
0
5
10
50
100
500
1000
2500
5000
LPS (ng/mL)

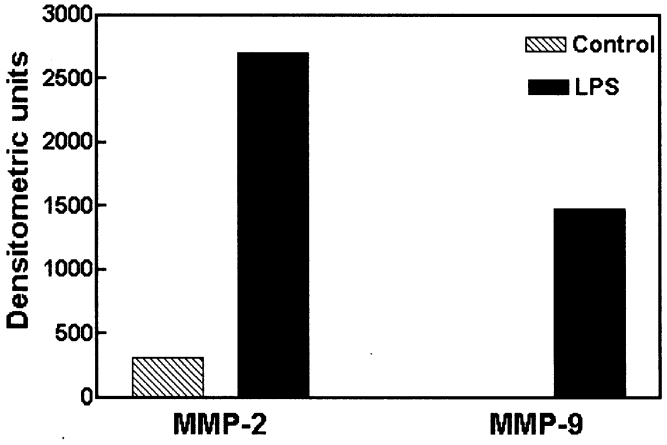
**Figure 4.4: LPS-mediated MMP expression by human monocytic cells**

Human MM6 cells ($10^6$ per well) were stimulated with 100 ng/mL LPS for 24 h. Cell-free media (9 µL) was mixed with 9 µL of the sample buffer and heat treated for 5 min. Soluble gelatinases released into the media were resolved by SDS-PAGE on a 10% gelatin gel. (**A**), Shown is a typical zymogram gel stained with Coomassie Blue, as detailed in Section 2.2.18. Lane 1, untreated MM6 cells; lane 2, LPS-treated MM6. (**B**), Level of expression of gelatinases MMP-2 and MMP-9 seen in **A** was quantified by densitometry. The histograms show expression intensity 24 h after stimulation with LPS.

A


UT
LPS
MMP-2
MMP-9

B


Densitometric units
3000
2500
2000
1500
1000
500
0
Control
LPS
MMP-2
MMP-9

expression, as quantified by densitometry. As evident, LPS caused a 5-6 fold upregulation in the constitutive MMP-2, and dramatic induction of MMP-9 following 24 h of stimulation. The response was also time dependent in that the induction of MMP was even stronger at 48 h.

## 4.4 SUMMARY AND DISCUSSION

In this chapter we have presented evidence for a strong TLR4 expression on human MM6 cells, suggesting that the lack of NOS II induction and NO production by human monocytic cells following LPS activation is not likely to be due to the absence of TLR-4. Although not investigated here, CD14 (both membrane-bound and soluble) has been found on these cells, as reported (Labeta *et al.*, 1993). Thus, it appears that from considering the receptor presence, the cells would be able to recognize the bacterial product. Subsequent studies presented here have shown that upon activation with LPS, MM6 cells were able to release large amounts of TNF-α (several fold higher than that by resting cells). In addition, exposure to LPS also triggered a significant

induction of MMP-9, as well as a substantial up-regulation (approximately five-fold higher than the constitutive expression) of MMP-2 in these human monocytic cells, as detected by zymography. The induction of the two gelatinases also appeared to be time dependent in that 48 h stimulation resulted in an even stronger

expression of the two MMP by MM6 cells than that seen after 24 h activation.

As the endotoxin used in these studies was derived from the same strain of *E. coli* as that used earlier for the NO investigation, the lack of detection of NO production by the human monocytic cell lines tested (MM6, U937, and THP-1) (Chapter 3) could not have been due to inherent unresponsiveness of the human cells to this particular strain of the bacteria. Therefore, the lack of NO production in human LPS- stimulated cells would likely be due to some blockage along the signalling pathway leading to gene activation.

# CHAPTER FIVE

# PKC-ETA IS REQUIRED FOR LPS-INDUCED EXPRESSION OF THE INDUCIBLE NITRIC OXIDE SYNTHASE IN HUMAN MONOCYTIC CELLS

## 5.1 INTRODUCTION

Our data from Chapter 3 have shown convincingly that human monocytic cells stimulated with endotoxin do not produce NO. The biochemical mechanisms underlying this phenomenon were not clear. However, several lines of evidence have supported a role of PKC in the LPS-triggered signalling pathway leading to production of NO in murine monocytic cells. For example, Severn and colleagues (1992) have shown that the PKC inhibitor Ro31-8220 significantly suppressed NO produced by murine J774A.1 following their activation with IFN-γ. Ro31-8220 was also shown to effect suppression of NOS II and PKC activity in murine monocytic cells from Raw 264.7 treated with LPS, suggesting that PKC might have an obligatory role in the regulation of NOS II by LPS (Paul *et al.*, 1995). In addition, Sodhi and Kumar (1994) provided evidence for involvement of other PKC inhibitors in the NOS II induction by LPS in murine peritoneal macrophages. H7 and chelerythrine chloride were found to significantly inhibit NO synthesis by peritoneal LPS-treated macrophages. Studies with PKC isozyme-specific antisense-oligonucleotides (Fujihara *et al.,* 1994; Chen *et al.*, 1998 A) and PKC activator PMA (Severn *et al.*, 1994) demonstrated an important role of PKC in the transcription of NOS II in murine monocytic cells.

### 5.1 Rationale and aims

Findings such as the ones discussed above prompted us to explore further what appeared to be a connection between PKC and NO production. Specifically, we wanted to determine if the absence of one or more PKC isotypes might be responsible for the inability of human monocytic cells to make NO following *in vitro* stimulation with LPS. Given that PKC has a large family, the first step would be to conduct a complete comparative expression of such PKC isotypes in both human and murine monocytic cells. Such analysis was important as there had been little information available regarding PKC isotype expression in monocytic cells. In a few studies where the expression was looked at, the investigations were often limited to one or two selective isoenzymes in murine (St. Denis *et al.*, 1998; Chen *et al.,* 1998; Diaz-Guerra *et al.,* 1996) or human (Monick *et al.*, 1998). Therefore, we first compared and contrasted PKC expression in monocytic cells from various human and murine cell lines, as well as those of human primary cultures. The findings from this investigation, as revealed later, form a basis for the work constituting the latter part of this chapter. Specifically, it deals with the effect of PKC transfection in a human cell line, studies on NOS II induction, and NO production by these monocytic cells following the transfection.

Majority of the data presented in this chapter has been published in the journal of *Nitric Oxide: Biology and Chemistry* (Pham *et al.*, 2003a).

## 5.2 METHODS

### 5.2.1 Assessment of PKC protein by western blotting and mRNA expression by RT-PCR

Monocytic cells grown in suspension or by adherence to plastic were extracted for total homogenates, as described (Section 2.2.10.1). Resulting cell lysates (110 μg) were subjected to acrylamide gel electrophoresis followed by western blotting (Section 2.2.11) with isotype-specific anti-PKC antibodies. Equal amounts of lysate prepared from fresh rat brain tissue were analyzed in parallel as a positive control.

Expression levels of various PKC isotypes were semi-quantified by a Chemi Imager (Alpha Innotech Corp.). Since they were all differentially expressed, the most abundant isotype was given a score of 100 and chosen to be a reference. Expression levels of all others were then standardized against this isotype.

In certain experiments where mRNA levels of a particular PKC isotype were desired, total RNA (Section 2.2.7) was prepared and subjected to RT-PCR with isotype-specific PKC primers (Section 2.2.8)

### 5.2.2 Cell transfection

MM6 cells were transfected with expression plasmid pks1.PKC-η, which

contains the full length PKC-η cDNA placed under the control of CMV/T2 promoter by LipofectAMINE. This promoter is useful as it is active in almost all mammalian cells. Briefly, cells were harvested and washed once with Opti-MEM I Reduced Serum Medium. The cells ($3 \times 10^6$ cells suspended in 0.8 mL of OPTI-MEM media) were plated in each well of the six-well plate and placed in a 37 $^0$C incubator. Meanwhile, DNA and LipofectAMINE solutions were prepared as follows. For each transfection, 2 µg DNA and 3 µL LipofectAMINE were added to separate tubes, each of which was made up to 100 µL with OPTI-MEM media. The two solutions were gently mixed, combined, and incubated at RT for 45 min to allow for the formation of DNA-liposome complexes. Subsequently, the complexes were carefully added to the cell suspensions, and the plate swirled gently to ensure uniform distribution. After 4 h incubation at 37 $^0$C, the cells were supplemented with complete growth media and cultured for another 48 h. Total RNA or cell extracts were recovered for RT-PCR and western blotting, respectively.

### 5.2.3 Assessment of NO production in PKC-η transfected MM6 cells following LPS and cytokine stimulation

Following transfection, $3 \times 10^6$ cells (per well) were seeded in a 6-well plate, cultured for 3 h, and treated with various doses of LPS and IFN-γ for 48 h. Total cell extracts were prepared for evaluation of NOS II protein by western blotting. Cell-free supernatants were accordingly analyzed for nitrite.

On some occasions, PKC-η transfected MM6 were treated with TNF-α and/or IFN-γ in the presence of LPS. Cell cultures were then measured for NO levels.

## 5.3 RESULTS

### 5.3.1 Expression of PKC isoenzymes in human and murine monocytic cells evaluated by western blotting

Using PKC isoform-specific antibodies as a research tool, we assessed expression of the isotypes in the three human and two murine cell lines, as well as in human MDM generated from PBMC of healthy individuals (Fig. 5.1). As shown, some isotypes were expressed more abundantly than others, and this was true for all cases tested: rat brain (positive control), human and murine monocytic cells. For example, as depicted in Table 5.1, PKC-θ was the most weakly expressed isotype overall. This was observed across the cell lines, human MDM, and the rat brain. On the other hand, PKC-ε was the most predominantly expressed, but this was only in the case of the rat brain. Taking advantage of its strongest expression, we used PKC-ε in the brain as a reference (100%) against which expression levels of all other isoforms were to be normalized. The results were then expressed as a percentage of this reference. As evident, all but one isoform (PKC-η) were present in larger quantities in the brain than in the monocytic cells, of either human or

**Figure 5.1: Protein expression of PKC isoenzymes in human and murine monocytic cells**

Total cell homogenate (110 µg) prepared from murine (J774A.1 and Raw264.7) and human (U937, THP-1, and MM6) monocytic cell lines were subjected to a western blotting analysis (Section 2.2.11) for evaluation of PKC isotype expression. Equal amounts of a rat brain sample were analyzed in parallel and used as a positive control. Shown is a representative western blot image from such analysis. Positive signals showed bands of the expected sizes for PKC-µ (mu) and PKC-ζ (zeta).

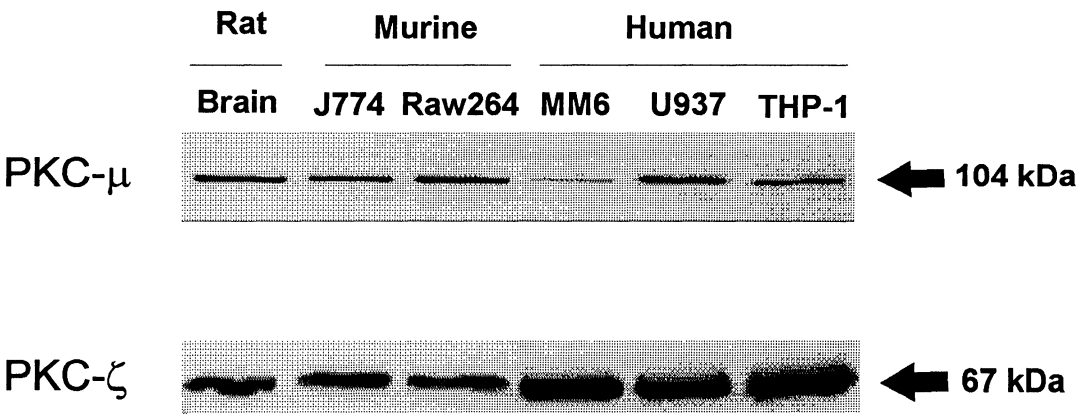
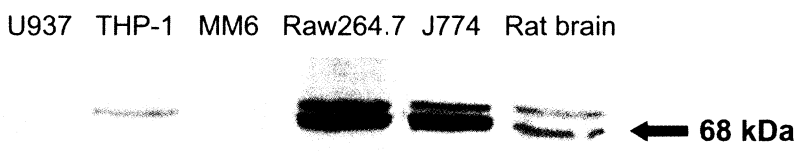

Rat
Murine
Human
Brain
J774
Raw264
MM6
U937
THP-1
PKC-μ
104 kDa
PKC-ζ
67 kDa

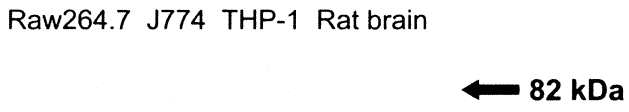
**Figure 5.2: Differential PKC-η expression in human and murine monocytic cells**

(**A**), Total cell extracts (110 µg) isolated from human (U937, THP-1 and MM6), murine (Raw264.7 and J774A.1) monocytic cells and rat brain were subjected to western blotting with a rabbit polyclonal anti-PKC η antibody (Section 2.2.15.1). (**B**), Binding specificity was ensured by incubating the antibody with excess blocking peptide prior to probing of the samples. The expected size of putative PKC-η is 68 kDa. The 82 kDa band present in THP–1 in **B** is an unknown species.

**A**


U937 THP-1 MM6 Raw264.7 J774 Rat brain
68 kDa

**B**


Raw264.7 J774 THP-1 Rat brain
82 kDa

murine origin. In addition, PKC-γ was not expressed in any of the five cell lines; this was consistent with the notion that this particular subtype is exclusively expressed in the brain (Nishizuka, 1984). Quantitatively, PKC-α, -β2, -δ, -θ, -ι, -ϵ, and -μ essentially showed similar expression levels in human and murine monocytic cells. There was some degree of variations in the levels of PKC-ζ expression among those tested. The differences, however, were not along the species line in that this isoform was present at a slightly higher level in human MM6 and U937 than MDM, THP-1 or their murine counterparts. The most intriguing finding of our screening lies with PKC-η and PKC-β1. PKC-η was abundantly expressed in murine cells, but completely deficient in the human cells. In contrast, PKC-β1 was barely detected in human monocytes and not found in murine cells.

In the case of PKC-η, the antibody failed to recognize the cognitive antigen in U937 and MM6 cells, while it barely reacted to THP-1 to show a weaker band of approximately 68 kDa, which is within the expected size of PKC-η (Fig. 5.2 A). In marked contrast, murine monocytic cells Raw 264.7 and J774A.1, as well as the positive control rat brain, each showed two very strong bands of 68 and 82 kDa when probed with the same antibody, with the 68-kDa entity being the more abundant species (Fig. 5.2 A). To confirm binding specificity, identical samples were probed with the PKC-η antibody in the presence of an antibody specific peptide. As shown in Figure 5.2 B, the presence of the competing peptide resulted in complete blocking of antibody binding to its cognate antigen in the rat brain and

**Table 5.1: Quantitative PKC isotype expression in human and murine monocytic cells as detected by western blotting**

[a]Isotype expression was quantified by densitometry with PKC from positive control rat brain. PKC-ϵ in the rat brain was most abundantly expressed, and thus given the highest score of 100. Expression levels of the other isotypes were then standardized against that of PKC-ϵ. The results are expressed as mean expression % of the epsilon isotype (ϵ) ± SD (number of experiments).

[c]Not done. Expression in both murine and human monocytic cells have been reported elsewhere (Ono *et al.*, 1989; Diaz-Meco *et al.*, 1994).

[d]Absence of PKC-η in human monocytic cells was not due to species specificity of the antibody as it was able to recognize the antigen in the human brain (Fig. 5.3).

## Table 5.1: Quantitative PKC isotype expression in human and murine monocytic cells as detected by western blotting[a]

| Isoform | Murine | | Human | | | | Rat |
|---|---|---|---|---|---|---|---|
| | Raw264.7 | J774 | THP-1 | MM6 | U937 | MDM | Brain[b] |
| Alpha (α) | n/d[c] | n/d | 54.9 ±5 (2) | 45 ± 9 (4) | 49.2 ± 10 (4) | 45.3 ± 5 (3) | 45.1 ± 2 (3) |
| **Beta 1 (β1)** | **0 (4)** | **0 (4)** | **14.1 ± 2 (2)** | **14.3 ± 4 (4)** | **20.6 ± 2 (4)** | **16.1 ± 3 (3)** | **42.0 ± 1 (3)** |
| Beta 2 (β2) | 35.8 ± 9 (4) | 31.7 ± 8 (2) | 54.2 ± 13 (3) | 44.3 ± 14 (4) | 44.0 ±11 (4) | 43.2 ± 7.6 (3) | 92.6 ± 14 (3) |
| Gamma (γ) | 0 (2) | 0 (2) | 0 (2) | 0 (2) | 0 (2) | 0 (2) | 88.7 ± 2 (2) |
| Epsilon (ε) | 8.3 ± 7 (3) | 10.2 ± 4 (3) | 10.6 ± 3 (2) | 36.3 ± 22 (4) | 24.9 ± 4 (4) | 26.3 ± 12 (3) | 100 ± 11 (3) |
| Delta (δ) | 23.1 ± 5 (2) | 26.1 ± 7 (2) | 23.1 ±5 (2) | 27.1 ± 6 (3) | 23.2 ± 6 (3) | 22.3 ± 5.0 (3) | 91.4 ± 6 (3) |
| Mu (μ) | 15 ± 18 (2) | 18.1 ± 7 (2) | 23.0 ± 2 (2) | 11.1 ± 6 (2) | 18.8 ± 4 (2) | 16.0 ± 4 (2) | 21.4 ±12 (2) |
| Theta (θ) | 11.1 ± 1 (3) | 9.6 ± 1 (3) | 5.6 ± 2 (2) | 9.0 ± 1 (3) | 7.9 ± 2 (3) | 6.9 ± 2 (3) | 13.1 ± 1 (3) |
| **Eta[d] (η)** | **142 ± 6 (4)** | **146.5 ± 2 (2)** | **0 (2)** | **0 (3)** | **0 (3)** | **0 (3)** | **83.7 ± 4 (3)** |
| Iota (ι) | 18.4± 2 (4) | 13.7 ± 2 (2) | 22.3 ±2 (2) | 26.4 ± 6 (4) | 22.9 ± 6 (4) | 24.4 ± 3 (3) | 38.8 ± 3 (3) |
| Zeta (ζ) | 49.6± 8 (2) | 43.0 ± 7 (2) | 54.5 ±10 (2) | 93.1± 19 (2) | 71.9 ± 22 (2) | 56.3 ± 5 (2) | 60.9± 11 (2) |

murine monocytic cells (Raw 264.7 and J774A.1) evident by the disappearance of the two bands. However, the peptide failed to block binding of the antibody to the single THP-1 band seen in Figure 5.2 A. To address the possible concern that the absence of PKC-η seen in the human cells were likely to be a consequence of inherent species specificity of this antibody and subsequent differences in epitope recognition, PKC-η expression was tested against fresh human brain tissue (kindly provided by Dr. Simon Avis, Memorial University) by western blotting using the same antibody. As shown in Figure 5.3, the antibody recognized the putative antigen in the human brain, as it did with murine monocyte J774A.1 and rat brain. This suggests that the absence of PKC-η in human monocytic cells was not due to the failure of the antibodies, which were used in our studies, to recognize the antigen.

### 5.3.2 Evaluation of PKC-η isoenzyme transcription in human and murine monocytic cells by RT-PCR

To explain whether the absence of PKC-η observed was due to the lack of expression of the protein or the inability of the antibody to recognize the antigen, we examined the levels of PKC-η gene transcription by RT-PCR. As shown in Figure 5.4, no PKC-η mRNA was found to be present in human monocytic cells, either MM6 or MDM cells. The plasmid pks1.PKC-η encoding the full length human

**Figure 5.3: Expression of PKC-η in human brain tissue**

Total cell extracts (110 µg) from human brain, murine monocyte J774A.1, and rat brain were subjected to western blot analysis with a rabbit polyclonal anti-PKC-η antibody, as described (Section 2.2.15.1). Shown is the result from a representative experiment.

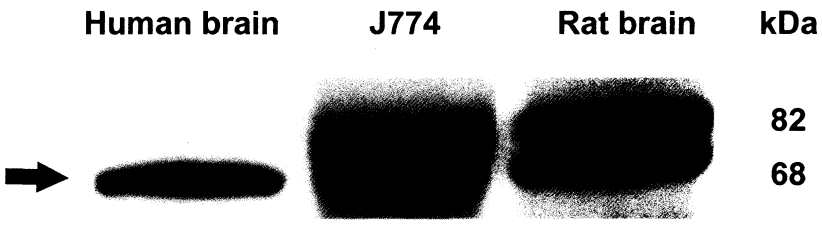
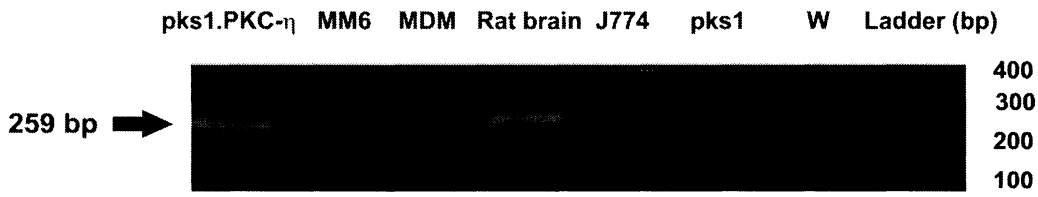

Human brain
J774
Rat brain
kDa
82
68

**Figure 5.4: Absence of PKC-η mRNA in human monocytic cells**

Total RNA (2-4 μg) harvested from human (MM6 and MDM) or murine monocytic cells (J774) as well as that from rat brain were analyzed by RT-PCR for PKC-η expression using gene specific primers. Samples containing water (W) in place of test cDNA and empty plasmid (pks1) were prepared in parallel as negative controls. The plasmid pks1.PKC-η encoding full length PKC-η was used as a positive control. PCR products (9 μL) were fractionated on a 1% EtBr-containing agarose gel. Positive signals showed the band of expected size of 259 bp.


pks1.PKC-η
MM6
MDM
Rat brain
J774
pks1
W
Ladder (bp)
259 bp
400
300
200
100

cDNA was used as a positive control.

### 5.3.3 Transfection of MM6 cells with PKC-η

In light of the findings in Figure 5.4, which showed that PKC-η mRNA was not present in MM6, we proceeded with the transfection of these cells with plasmid pks1.PKC-η. This plasmid is a CMV (cytomegalovirus)-driven expression vector encoding the full length PKC-η cDNA MM6 was transfected with the vector using lipoFECTAMINE. After 48 h, the cells were assessed for PKC-η protein expression by western blotting. Figure 5.5 shows a typical result from a representative experiment. It demonstrates the presence of PKC-η isoenzyme (68 kDa) in MM6 cells transfected with the plasmid encoding PKC-η and in both the murine J774A.1 and rat brain (positive controls), but not in untransfected MM6 cells cells or those transfected with the empty vector.

### 5.3.4 Effect of PKC-η on NOS II expression and NO production by human monocytic cells

As illustrated in Figure 5.6 A, NOS II enzyme was not expressed at the lowest LPS dosage tested, 0.1 μg/mL by MM6 cells transfected with PKC-η (psk1.PKCη). Marked upregulation of NOS II was seen at 1 μg/mL LPS with no further augmentation of the enzyme at higher LPS concentrations (up to 10 μg/mL).

**Figure 5.5: Expression of PKC-η in human monocytic cells following transfection**

(**A**), MM6 cells ($2.5 \times 10^6$) were transfected with 3 µg of pks1.PKC-η (MM6-T) or an empty vector (pks1) in the presence of 4 µg LipoFECTAMINE, as described (Section 2.2.16). MM6 cells that were not transfected (MM6-UT) served as a negative control while murine J774 cells and rat brain served as positive controls. After 48 h, total cell extracts were prepared, and the samples (110 µg) probed for PKC-η by Western blotting using the PKC-η-specific antibody (Section 2.2.15.1). (**B**), To demonstrate binding specificity, an identical western blot was prepared and probed with the same PKC-η antibody previously incubated with the PKC-η competing peptide. Three independent experiments gave identical results.

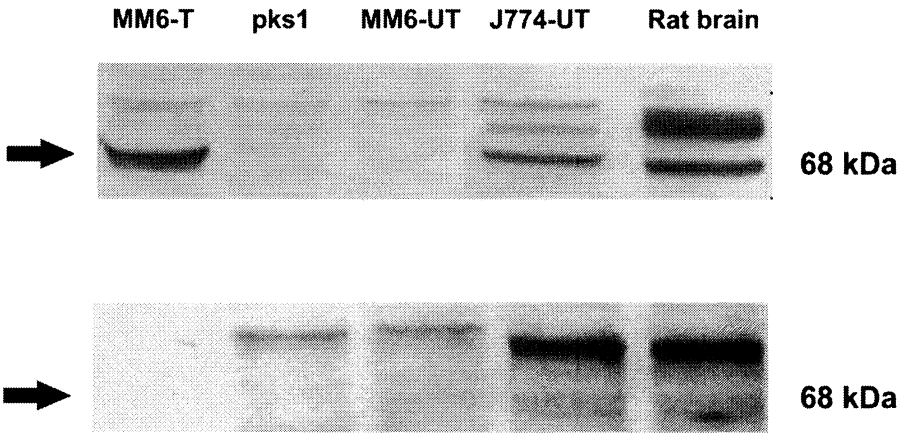
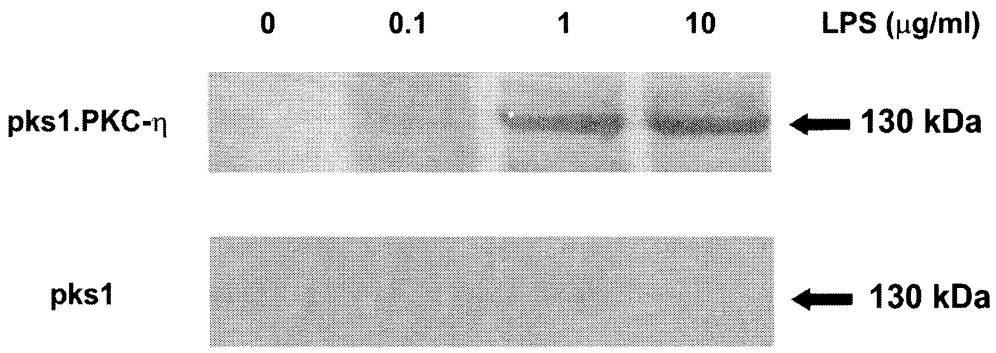

MM6-T
pks1
MM6-UT
J774-UT
Rat brain
68 kDa
68 kDa

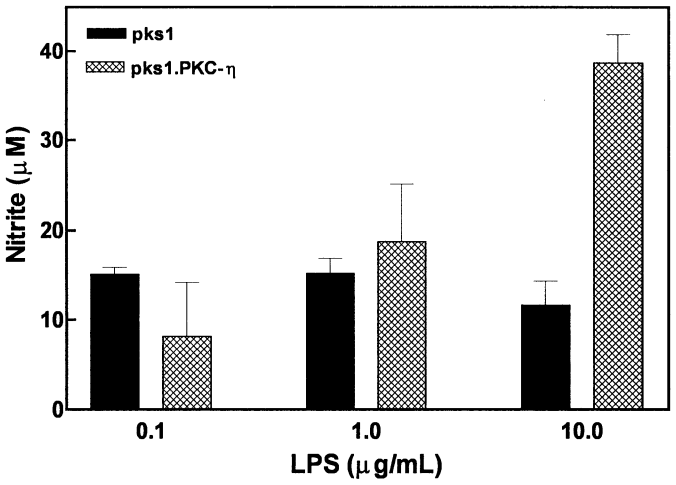
**Figure 5.6: Effect of PKC-η on NOS II expression and NO production in human monocytic cells following stimulation with LPS and cytokines**

Human MM6 cells were transfected with either the empty vector (pks1) or plasmid encoding PKC-η (pks1.PKC-η), as described (Section 2.2.10). The mock transfected cells treated in the same way as the PKC-η expressing cells served as a negative controls. After the transfection, 2 mL (per well) of 1.5 x $10^6$ cells/mL was seeded in a 6-well plate, and incubated for 3 h. Subsequently, the cells were stimulated with varying concentrations of LPS for 48 h and harvested for the preparation of total cell homogenate. (**A**), Total cell lysate (110 µg) was analyzed for NOS II by Western blotting. (**B**), Cell-free supernatant (100 µL) was collected for nitrite measurements by Griess assay. (**C**), In some experiments, PKC-η expressing MM6 cells were also treated with TNF-α and/or IFN-γ, in addition to LPS, and cell culture media analyzed for NO contents. NO, if any, released by the untreated cells was used as a background. NO values were expressed as mean ± SD of three triplicate experiments after the background had been subtracted. On the western blot images, the positive signals showed a 130-kDa band, the expected size of NOS II.

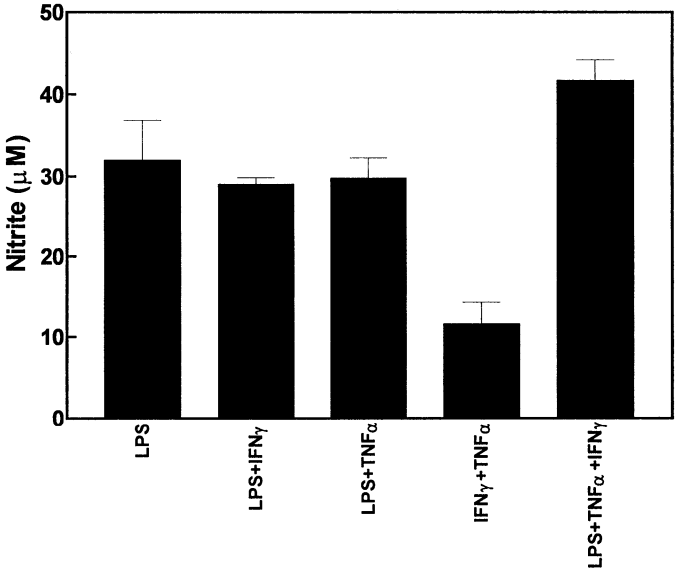
A



B


pks1
pks1.PKC-η
Nitrite (μM)
0
10
20
30
40
0.1
1.0
10.0
LPS (μg/mL)

C


Nitrite (μM)
0
10
20
30
40
50
LPS
LPS+IFNγ
LPS+TNFα
IFNγ+TNFα
LPS+TNFα+IFNγ

In contrast but as expected, NOS II was not found to be expressed at any of the LPS doses tested in MM6 cells transfected with the empty plasmid (pks1). The enzyme induced by the human MM6 cells expressing PKC-η was functional, as shown by the production of NO (Fig. 5.6 B). However, unlike the synergistic effects of cytokines and LPS observed earlier in murine monocytic cells (Section 3.2.1), similar co-stimulation with LPS and cytokines (TNF-α or IFN-γ) did not induce any increase in the levels in LPS-mediated NO production by these transfected human cells (Fig. 5.6 C). It was only when the PKC-η expressing MM6 cells were co-treated with LPS and a cytokine combination of TNF-α/IFN-γ, did we observe slight augmentation of NO released. However, the enhancement was 10-15% of that seen in the murine cells (Section 3.2.1).

It is not possible that the NOS II expression and NO induction observed in our experiments was due to PKC-η expression in a few isolated transfected cells as the same number of J774A.1 cells and the transfected MM6 cells were used to prepare total cell extracts, of which the same amounts (110 μg) were analyzed, and similar staining of bands observed.

## 5.4 SUMMARY AND DISCUSSION

It is clear from our study that of eleven isoforms known to exist in the PKC superfamily, only two make human and murine monocytes differ. First, PKC-η was

grossly expressed in the two murine monocytic cell lines J774A.1 and Raw 264.7, but completely absent from the three human cell lines tested (U937, MM6, and THP-1). Second, PKC-β1 was minimally expressed in the human monocytic cells but was completely absent in their murine counterparts. Because there exists a possibility of some unknown factors being spontaneously present in transformed cell lines that could alter expression of different genes including PKC, our observations regarding the unique distribution of PKC-η and PKC-β1 may not necessarily reflect the in vivo situation. To address the concern, we screened for different isotypes of PKC from MDM isolated from healthy human subjects, and found that the expression in this instance was identical to that of the cell lines, both qualitatively and quantitatively (Table 5.1).

A literature search on a link of these isoforms with NO capability in monocytic cells was not very fruitful. The work by Chen and colleagues (1998, A and B) with antisense oligonucleotides has revealed that PKC-η was involved in the expression of NOS II in murine astrocytes, but that the same isoform had no role in the regulation of NOS II gene in murine monocytic cells from the Raw 264.7 cell line following 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulation. Clearly, these findings needed to be further assessed, and more work needed to be carried out if one were to achieve better understanding of this very complex pathway. With Chen using the approach of "deleting" out the PKC-η isoform in murine monocytic cells by employing the antisense oligonucleotides, it was of interest to us to tackle

the issue directly from the human model, as observations made in the murine system may not necessarily be applicable in human situation. In addition, we wanted to ascertain if Chen's findings were specific to TPA stimulation or they were also true with other stimuli such as LPS. With this aim, we proceeded with transfection of the gene to the human monocytic cell line MM6. Our findings from the transfection studies (Fig. 5.6) have shown for the first time that human monocytic cells transfected with PKC-η are capable of expressing NOS II leading to production of NO at levels that could be readily detected in cell culture media. This was in marked contrast to what was previously thought (Denis, 1994). The evidence here suggests that PKC-η may play an important role in regulating NOS II gene expression in human monocytic cells. However, as alluded to earlier, these findings are in contrast with Chen's work (1998 B) which suggested that long-term treatment of TPA did not seem to effect down-regulation of PKC-η like it did with other PKC isoforms they tested. The assumption underlying the work was that PKC-η would respond to the TPA treatment, and this raises a question of whether or not this is indeed true in all instances. Secondly, in this study the NF-κB translocation after LPS stimulation was not measured at various time points. Therefore, it is equally possible that the chosen time point might not have been long enough to allow for the activation and/or translocation of PKC-η to the membrane, albeit this seems unlikely.

In murine monocytic cells, expression of the NOS II gene is transcriptionally

regulated by a basal promoter known as Region I, and by an enhancer element known as Region II. Region I is responsive to LPS while Region II is essentially IFN-γ responsive (Lowenstein *et al.*, 1993). The work done by Zhang *et al.* (1996) has shown that the human counterpart Region II shares an approximate 76% similarity with that of the murine, and that the nucleotide substitutions within this region may be the reason for hyporesponsiveness of human monocytic cells to LPS. Their work with chimeric human/murine promoter constructs revealed that the response to LPS was qualitatively the same as that of the murine construct. Quantitatively, however, the response of the chimeric construct was only half of the wild-type construct supporting their hypothesis that Regions I and II of the human promoter are not the only regions involved in regulating the expression of NOS II, and that there may be other signals needed to fully induce NOS II gene expression. Our current work supports this aspect of the hypothesis as at a given concentration of LPS, NO production by the human cells was approximately 60% of that seen in murine cells.

Novel findings presented here warrant further research to delineate cross-talk between PKC-η and other signalling molecules which are involved in the LPS-mediated NOS II signalling pathway in human monocytic cells. If a positive correlation between NOS II and PKC-η were to be established in certain disease processes, it would be plausible to envision that inhibitors to PKC-η would offer potential therapeutic benefits of inflammatory conditions where inappropriate

release of NO is known to contribute to their pathologies.

# CHAPTER SIX

# EFFECTS OF TRIPTERYGIUM (TwHf) DERIVATIVES ON THE *IN VITRO* PRODUCTION OF INFLAMMATORY MEDIATORS BY MONOCYTIC CELLS

# 6.1 INTRODUCTION

## 6.1.1 Current management approaches for rheumatoid arthritis

Currently, there are a number of conventional therapeutic approaches for the treatment of RA. The initial drug treatment for RA after the onset of disease involves the use of salicylates, nonsteroidal anti-inflammatory drugs and selective cyclooxygenase 2 (COX-2) inhibitors (rofecoxib) to reduce pain and improve motion (Geba *et al.*, 2002). Low dose glucocorticoids (*e.g.*, prednisone) are effective in relieving inflammation in patients with active RA by interfering with leukocyte trafficking, proliferation, and function (De *et al.*, 2002). These drugs, however, do not have a role in preventing the progression of disease, and require additional help from a group of compounds known as disease-modifying anti-rheumatic drugs (DMARD). Four of the most widely used DMARDs are Methotrexate (MTX), antimalarial, sulfasalazine, and hydroxy-chloroquine which can all reduce disease progression owing to their anti-inflammatory and anti-proliferative effects. Although less used in the last decade, various gold salt compounds, including sodium aurothiomalate and auranofin, have also been ultilized for the treatment of RA (Hashimoto *et al.*, 1994). These gold salts are thought to interfere with the formation of synovial blood vessels (neovascularization), thus significantly minimizing infiltration of immune cells and impeding growth of the sinovial pannus (Saura *et al.*, 1994). With respect to the involvement of cytokines in RA, it has now been shown that TNF-$\alpha$ and IL-1$\beta$ are arthritogenic, and both are critical in

initiating and perpetuating the inflammatory process leading to joint destruction (Dayer, 2002; Bingham, 2002). Studies from arthritic patient samples obtained *ex vivo* have lead to the development of monoclonal antibodies against TNF-α (*e.g.,* infliximab and cA2) as possible therapeutic interventions for RA. Infliximab has now been approved for yet another type of medication for RA treatment. However, as powerful as some of these compounds might be, they all have different side effects, with some being more severe than others. Included in this list are neural (Sicotte and Voskuhl, 2001), gastrointestinal (Thomas *et al.*, 2002), renal (Fukuchi *et al.*, 1998), and respiratory complications (Blancas *et al.*, 1998). It is also expected that RA therapies which target soluble inflammatory mediators such as TNF-α and NOS II may not prove efficacious for all patients. Clinical data, as supported by basic science studies, have revealed that in basically every approach, there are many patients who are unresponsive to the above mentioned treatments (Kalden, 2002). For those who do respond, adverse effects arising from these long term therapies are poorly documented. Thus, other therapeutic alternatives are needed for the disease.

### 6.1.2 Tripterygium *wilfordii* Hook f. (TwHf): a Chinese traditional medication with immunological activity

TwHf is a perennial vine that belongs to the *Celastracae* family. The plant

grows naturally in the mountainous southeastern regions of China, where it is also referred to as Lei Gong Teng Pian or Thunder god vine. For 2000 years, crude preparations of the plant root, thought to contain all the therapeutic components of the plant, have been used in traditional Chinese medication for the treatment of a number of conditions including fever, oedema, and joint pain. The exact mechanisms by which the herb works, however, are not clearly defined. This is partly because of the large number of natural compounds (more than 70) present in the root including diterpenes, triterpenes, glycosides and alkaloids although there is a great deal of similarity in the structures of many of these (Zhang *et al.*, 1990). To this date, the plant root is still used as a traditional medication for the treatment of a number of diseases including RA, systemic lupus erthyematosus (SLE), psoriasis, and more recently leukaemia. The medication is usually administered orally as either a ground power or a glycosidic extract.

In the past thirty years, much work has been devoted to refining the crude extracts of TwHf and purifying the components. Several different extracts including an aqueous concoction, an alcohol extract known as T1, a chloroform/methanol extract T2, and an alcohol/ethyl acetate (EA) fraction have been prepared, and explored for their anti-inflammatory and immunosuppressive effects *in vivo*. The chloroform preparation T2 was revealed to: 1) ameliorate type 2 collagen-induced arthritis in mice (Asano *et al.*, 1998), and 2) inhibit histamine-induced increase of vascular permeability in rats and delayed type hypersensitivity in guinea pigs

(Zheng *et al.*, 1983), while the EA extract was shown to suppress carrageenan-induced inflammation in rats (Tao *et al.*, 1999). The mechanism of action of these extracts have been studied *in vitro* with cell cultures either from cell lines or from samples obtained from RA patients *ex vivo*. These investigations have demonstrated various anti-inflammatory and immunosuppressive properties of these extracts. For example, the numerous glycosides of the T2 extract have been shown to dramatically inhibit proliferation of T lymphocytes as well as IL-2 and IL-4 production from activated T cells (Dong, 1997). As well, the glycosides have been documented to suppress production of IL-1, IL-6, IL-8, TNF-α, and prostaglandin E2 (PGE2) by monocytes/macrophages (Chang *et al.*, 1997) and secretion of IgG by B cells (Chang *et al.*, 1997). In addition to inhibiting IL-2 synthesis, the T2 extract has also been shown to abrogate the synthesis of PGE2 by mononuclear phagocytes (Chou *et al.*, 1998). Work by Mackawa and colleagues (1999) has revealed that the suppression of PGE2 by the glycosides of T2 was due to inhibition of COX-2 transcription via NF-*k*B. Similarly, Tao and colleagues (1998) observed the same phenomenon with the EA extract and purified triptolide, a diterpenoid triepoxide and a major constituent of the TwHf root. Both types of extracts and triptolide had no effects on either the activity or expression of the constitutively expressed COX-1, again reflecting the anti-inflammatory properties of the herbal medicine. In addition to suppressing the production of PGE2 by monocytic cells, triptolide has also been shown to suppress several cytokines

including IL-1 (α and β), IL-6 and TNF-α in the LPS-treated murine mononuclear phagocytes. In line with these inflammatory mediators, triptolide also attenuates the release of proMMP-1 and proMMP-3 (precursor of MMP) induced by IL-1β while augmenting the production of tissue inhibitor of MMP (TIMP)-1 and -2 in synovial fibroblasts (Lin *et al.*, 2001). Taken together, these extracts from TwHf affect production of various inflammatory mediators by immune cells including lymphocytes, macrophages, and fibroblasts. In so doing, they are able, through various mechanisms, to regulate the function of these cells, which are all involved in different capacities in the inflammatory process. This may be able to explain the observed high efficacy of the plant in ameliorating inflammatory conditions like arthritis.

With the support of 2000 years of clinical use in China, TwHf has rapidly gained attention within the scientific community in North America during the past 30 years. However, only relatively recently have the components been purified and chemically identified.

### 6.1.3 Chemical constituents of TwHf compounds used in the present investigation

Terpenoid compounds are the simplest naturally occurring isoprenoid compounds found widely in nature, especially in plants. All isoprenoid compounds have repeating five carbon atom units (isoprenes) in their carbon skeletons, with

terpenes containing two of these isoprene units joined together in a cyclic arrangement. Hence, a diterpene will have four and a triterpene will have six of these isoprene units. Using the same nomenclature, a terpenoid compound has multiples of the five carbon isoprene units.

### 6.1.4 Inhibitory effects of TwHf compounds on LPS-induced NO production by monocytic cells

Previously in Dr. Richardson's laboratory, seventeen TwHf compounds (provided by Dr. James Kutney), which were either natural or synthetic derivatives, were screened for their effects on: (1) cell toxicity and (2) the production of LPS-mediated NO by murine monocytic cells (Barrett, 1998). These natural compounds included: (1) diterpenes and triterpenes triptophenolide, (2) triptoquinone, (3) celastrol, (4) triptolide, (5) tripdiolide, and (6) tingenone, while the synthetic derivatives of TwHf included JPK-109 and JPK-101. The results from this work have shown that these compounds varied in toxicity and their ability to inhibit NO production by murine monocytic cells from J774A.1. The *in vitro* therapeutic index (TI) for NO ($TC_{50}/IC_{50}$ with the $TC_{50}$ being the dose where 50% of the cells die and the $IC_{50}$ being the concentration at which NO synthesis is suppressed by 50%) was 2.7 for triptophenolide, 5.1 for celastrol, 9.4 for JPK-109, and 4.4 for triptolide. It should be noted that although celastrol and triptolide demonstrated high potency,

they also conferred a cytotoxic effect on the cells tested. Thus, these three compounds may have potential as novel anti-inflammatory agents providing that the observed level of cytotoxicity could be appropriately addressed. In contrast, suppression of LPS-mediated NO synthesis by tripdiolide and tingenone was due to their toxicity on the murine monocytic cells, and not due to direct inhibition/suppression of the expression of NOS II enzyme. An important observation made from the study by Barrett (1998) was that the compounds themselves did not induce NOS II expression or NO production in these cells.

### 6.1.5 Aims

The work succinctly summarized above (Barrett, 1998) has provided important information on toxicity and the ability to suppress NO production by murine macrophages. However, it was not known from these studies as to whether the drugs caused inactivation of the NOS II enzyme or whether they played a role in the suppression of NOS II expression. It is with this in mind that follow-up experiments were done and a summary of the results are presented in this chapter. In addition, it was also of interest to explore potential effects of these compounds on two other important macrophage functions thought to be essential in the development and maintenance of the inflammatory process: synthesis of TNF-$\alpha$ and MMP. In these experiments three compounds, namely JPK-101, JPK-109, and JPK-113 were studied. JPK-101 and JPK-109 are synthetic diterpenes, while JPK-

113 (celastrol) is a quinone methide triterpene purified from the plant.

A manuscript containing the data reported in this chapter is in preparation for submission to a peer-reviewed journal.

## 6.2. MATERIALS AND METHOD

### 6.2.1. Materials

Stocks of JPK-101 and JPK-109 were dissolved in 20% reagent grade absolute ethanol, while that of JPK-113 was prepared in 5% DMSO. In all three cases, the solutions were used within 45 min following preparation.

### 6.2.2 Cell treatment with natural and synthetic derivatives of TwHf

Monocytic cells were treated with appropriate doses of the drug (dissolved in either reagent ethanol or 0.5% DMSO/PBS). After 45 min, unless stated otherwise, LPS was added (usually 1 µg/mL, unless specified to the contrary in figure legends), and the cells were cultured for 24 h or 48 h depending on the experiment. Cell-free supernatants or cells were prepared and analyzed, as appropriate.

In certain experiments where mechanism of action for a TwHf compound (JPK-109) was desired, a fixed concentration (3 µg/mL), earlier established to give maximum inhibition, of the drug was added to MM6 cells at various time points

before or after LPS addition. After 18 h, the cells were analyzed for NO and NOS II, as appropriate (see below).

### 6.2.3 Assessment of the effects of TwHf on the induction of NO, TNF-α, and MMP

After cells were treated as described above, they were harvested for extraction of total lysates, which were subjected to NOS II investigation by western blotting. Corresponding cell-free cultures were analyzed for: (1) nitrite by the Griess assay (Section 2.2.12.1), (2) TNF-α by the cytotoxicity bioassay with L929 (Section 2.2.13), and (3) MMP activity by zymography (Section 2.2.14). Samples of untreated cells or cells treated with LPS alone served as negative and positive controls, respectively.

### 6.2.4 Data analysis

Magnitude of the inhibitory effects of the TwHf compounds on NOS II and MMP were quantified by densitometry image analysis. A baseline (value from untreated cells) was subtracted in the analysis. In the case of NOS II, NO, and TNF-α, the background-subtracted value was normalized against a positive control (cells treated with LPS alone).

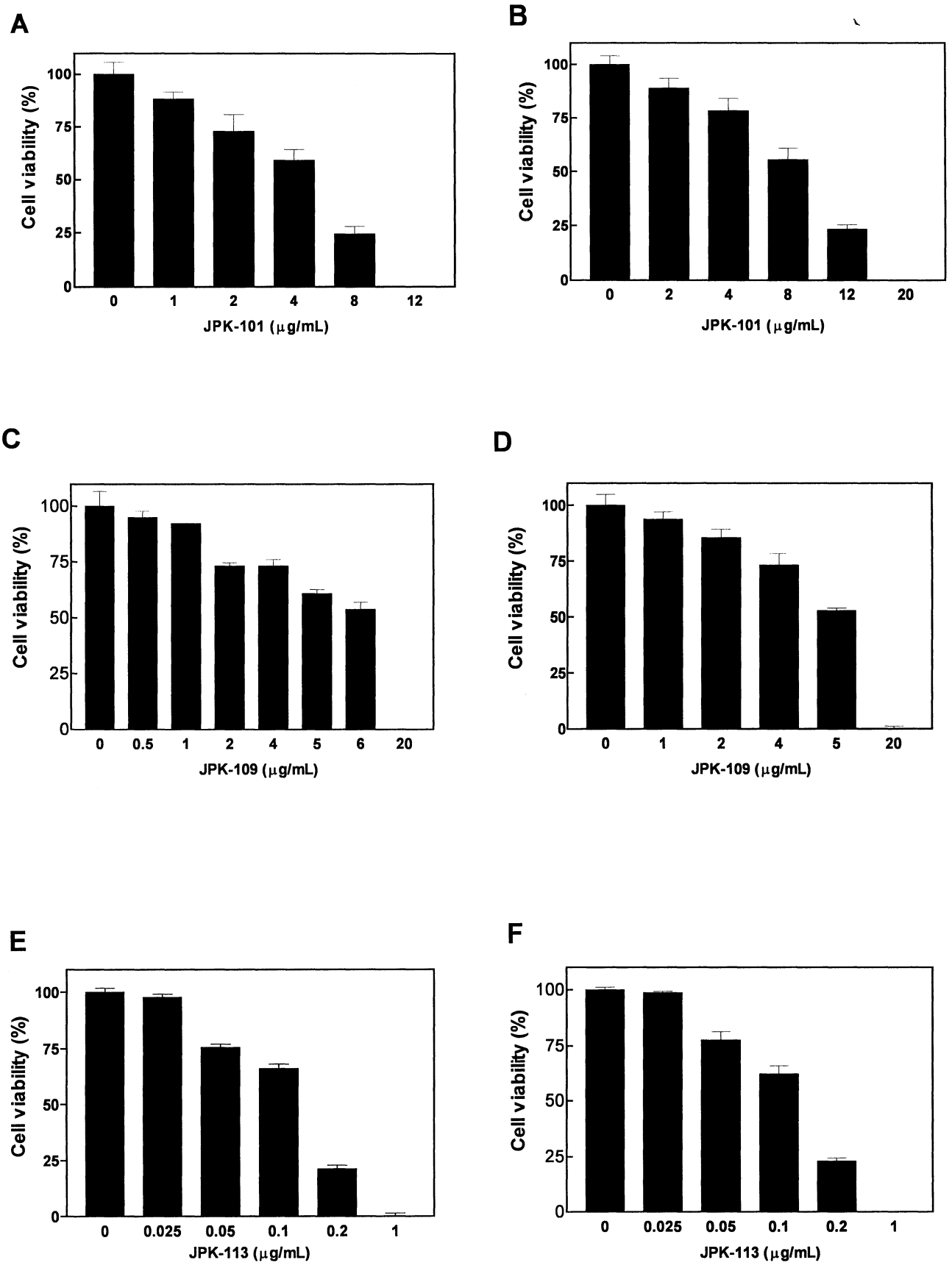
## 6.3 RESULTS

### 6.3.1 Toxicity studies

In any experiment that involves assessing the effect(s) of a novel compound on a particular cell function, it is essential to perform preliminary studies to determine its toxic doses on the cells being investigated. This piece of information is extremely important and unfortunately often ignored in many studies. Such practice can lead to inaccurate determination of the true effects of the drug. With this in mind, we investigated toxicity of the three TwHf compounds on human and murine monocytic cells from the cell lines MM6 and J774A.1, respectively by the MTT assay. As shown in Figure 6.1, in both J774A.1 (Fig. 6.1 A) and MM6 (Fig. 6.1 B), the toxic effect of JPK-101 started becoming obvious between 4-8 μg/mL. The $TC_{50}$ values were determined to be 6.1 ± 2.3 μg/mL for J774A.1 and 7.2 ± 0.8 μg/mL for MM6. Similarly with JPK-109, 50% of cell death was seen at 6.1 ± 1.2 μg/mL in J774A.1 (Fig. 6.1 C) and 5.1 ± 0.8 μg/mL (Fig. 6.1 D) in MM6. JPK-113 on the other hand appeared to be much more toxic in the two cell lines than JPK-101 and JPK-109 in that the $TC_{50}$ was 170 ± 35 ng/mL for murine J774A.1 (Fig. 6.1 E) and 150 ± 26 ng/mL (Fig. 6.1 F) for human MM6. Generally speaking, although the $TC_{50}$ values were different among the three compounds, the drugs appeared to exert similar toxicity on human and murine monocytic cells.

**Figure 6.1: Effects of TwHf compounds on cell viability as determined by the MTT assay**

J774A.1 (**A**, **C** and **E**) or MM6 (**B**, **D** and **F**) cells were plated at density $2x10^5$ per well in a 96-well plate. Drug solutions containing appropriate concentrations of JPK-101 (**A** and **B**), JPK-109 (**C** and **D**), or JPK-113 (**E** and **F**), were then added. After 45 min, the cells were supplemented with 1 µg/mL LPS, and left incubated for another 24 h. The MTT assay was then carried out, as described (Section 2.2.5.2). Untreated cells served as a negative control, and assumed to remain viable after the 24-h incubation period, while those that were treated with 2.5% DMSO were assumed to have been all killed at the same time point. The optical density values obtained from these two samples were used as the reference points for normalization of test samples. Data analysis was done using Graph Pad Prism software. In most cases, the expressed values were mean ± SD of three different experiments done in triplicate.


A
Cell viability (%)
100
75
50
25
0
0
1
2
4
8
12
JPK-101 (μg/mL)
B
Cell viability (%)
100
75
50
25
0
0
2
4
8
12
20
JPK-101 (μg/mL)
C
Cell viability (%)
100
75
50
25
0
0
0.5
1
2
4
5
6
20
JPK-109 (μg/mL)
D
Cell viability (%)
100
75
50
25
0
0
1
2
4
5
20
JPK-109 (μg/mL)
E
Cell viability (%)
100
75
50
25
0
0
0.025
0.05
0.1
0.2
1
JPK-113 (μg/mL)
F
Cell viability (%)
100
75
50
25
0
0
0.025
0.05
0.1
0.2
1
JPK-113 (μg/mL)

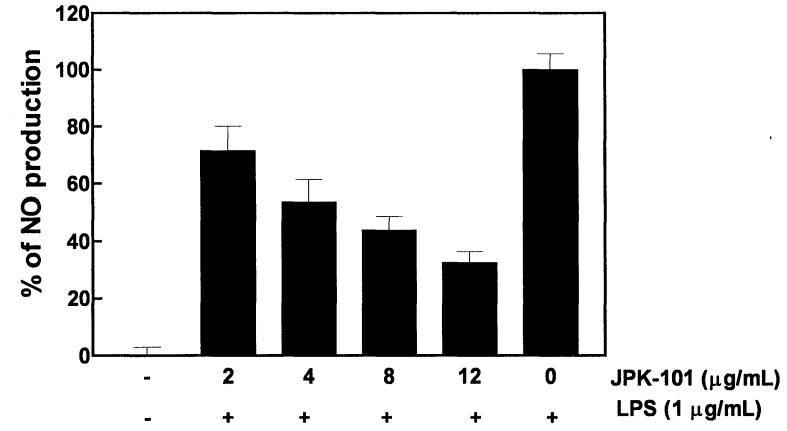
### 6.3.2 Effects of TwHf compounds on NO production by murine monocytic cells: A reassessment

Murine monocytic cells from the J774A.1 cell line were treated with increasing concentrations of the drugs for 45 min prior to being stimulated with LPS (1 µg/mL). Cell-free supernatants collected 24 h later were measured for NO by the Griess assay. As shown in Figure 6.2 A, the synthetic derivative JPK-101 inhibited LPS-induced NO production in a dose dependent manner. At 2 µg/mL, nearly 20% of NO production in murine J774A.1 was suppressed and the inhibition was steadily decreased at higher concentrations of NO. The $IC_{50}$ was determined to be 2.2 ± 0.4 µg/mL. A similar pattern of NO suppression was also seen in LPS-activated J774A.1 cells exposed to JPK-109 (Fig. 6.2 B). In this instance, JPK-109 appeared to be more effective than JPK-101 in that as little as 500 ng/mL of the compound was sufficient to reduce the NO synthesis by 30%. A complete inhibition was observed at 6 µg/mL of JPK-109. The $IC_{50}$ for this compound was approximately 1.05 ± 0.2 µg/mL. Similar screening for NO suppression of the natural TwHf celastrol (JPK-113) revealed that although the pattern was similar to that of the other compounds in terms of dose response, JPK-113 was less effective than the other two at inhibiting NO production ($IC_{50}$:75 ± 26 ng/mL). At the lowest dose tested (25 ng/mL), JPK-113 was found to mediate nearly 10% reduction in NO leading to 80% suppression at 250 ng/mL. However, given that the $TC_{50}$ for this compound in J774A.1 was previously determined to be 170 ng/mL (Fig. 6.1 E), the

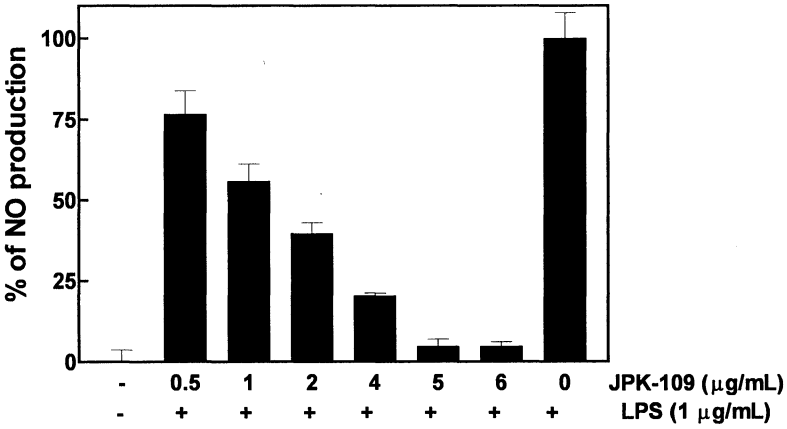
**Figure 6.2: Effect of TwHf compounds on LPS-induced NO production by murine J774A.1 cells**

J774A.1 cells (2.5 x $10^6$ per well) was seeded in a 60-mm petri dish, and cultured over night. Old medium was removed, and the cells were briefly washed with ice cold PBS. Fresh medium (2.5 mL) containing appropriate concentrations of the drug was added to the wells. The cells were stimulated with LPS (1 µg/mL) 45 min later for 18 h. Cell-free supernatant was measured for NO by the Griess assay, as detailed (Section 2.2.12.1). The cells were saved for a parallel western analysis (Fig. 6.3). The data were normalized to the control (cells treated only with LPS) using Graph Pad Prism software. (**A**), JPK-101; (**B**), JPK-109; and (**C**), JPK-113. Results were expressed as mean ± SD of four experiments done in triplicate.

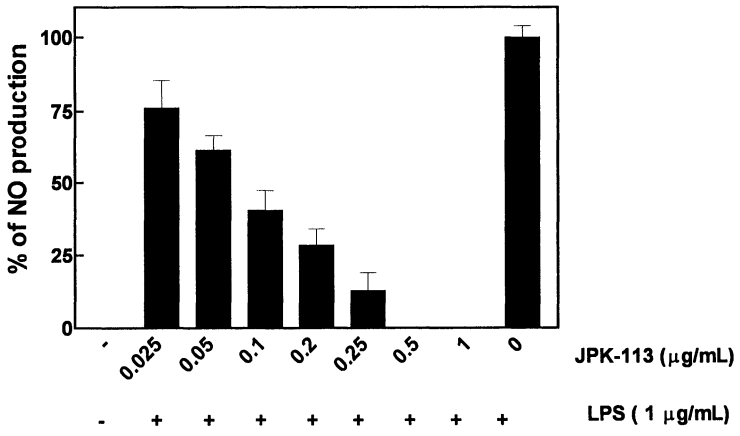
A


% of NO production
120
100
80
60
40
20
0
- 2 4 8 12 0
JPK-101 (μg/mL)
- + + + + +
LPS (1 μg/mL)

B


% of NO production
100
75
50
25
0
- 0.5 1 2 4 5 6 0
JPK-109 (μg/mL)
- + + + + + + +
LPS (1 μg/mL)

C


% of NO production
100
75
50
25
0
- 0.025 0.05 0.1 0.2 0.25 0.5 1 0
JPK-113 (μg/mL)
- + + + + + + + +
LPS ( 1 μg/mL)

dramatic inhibition at 250 ng/mL is not likely to have any biological significance as the results would probably be due to the presence of fewer NO producing cells (*i.e.*, an indirect consequence of cell death).

### 6.3.3 Effects of TwHf compounds on NOS II expression by murine monocytic cells

In an effort to decipher the mechanism whereby these three TwHf compounds suppressed NO production in J774A.1 cells, total cell extracts from the LPS-stimulated cells were analyzed by western blotting for NOS II display. The level of induction was normalized against that of LPS-activated J774A.1 cells, which were not treated with the drug. As illustrated in Figure 6.3 (A and B), at 1 μg/mL JPK-101 did not appear to have any suppressing effects on NOS II induction by J774A.1 cells. However, at higher concentrations, the synthetic compound began to show its inhibitory activity in that starting at 2 μg/mL, it gave rise to a gradual but steady decrease in NOS II expression at increasing JPK-101 doses. Suppression of close to 90% was observed at 12 μg/mL, the maximum concentration tested.

Similarly, JPK-109 also caused significant attenuation of the NOS II enzyme in LPS-treated J774A.1 cells (Fig. 6.3 C and D). In accord with the NO data presented earlier (Fig. 6.2 B), NOS II induction was suppressed by nearly 40% that of the control (LPS-stimulated cells in the absence of JPK-109) at 500 ng/mL, the lowest concentration tested. As also shown with JPK-101, the response was dose

dependent with NOS II expression decreasing as the drug dosage increased. Less than 18% of the NOS II expression remained at 6 µg/mL, the highest concentration used.

When JPK-113 was screened for its effects on the induction of NOS II by LPS in J774A.1 cells, a gradual decrease in NOS II expression was observed with approximately 10% of the inhibition seen at 25 ng/mL. It was clear from our studies that the decrease in NOS II synthesis became more pronounced with higher concentrations of JPK-113 leading to 50% inhibition at approximately 100 ng/mL (Fig. 6.3 E and F).

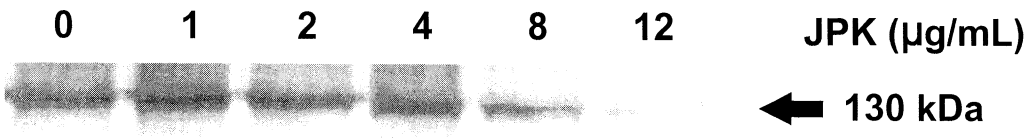
#### 6.3.3.1 Mechanism of action of JPK-109

The data obtained have revealed the suppressive effects of all three TwHf compounds on both NOS II expression and NO production in monocytic cells. In the next step, we wanted to explore further how one of these compounds JPK-109 works. There are several possible mechanisms by which the compounds may be acting: (1) blocking of the LPS receptor; (2) interfering with receptor signalling; (3) preventing gene transcription; (4) inhibiting translation of NOS II mRNA, and (5) blocking synthesis or interfering with function of NOS II and/or co-factors required by the enzyme. In an effort to gain some insight as to which of these mechanism(s) are likely to be involved in the observed inhibitory effect of JPK-109 on NOS II expression and NO production, we exposed the cells to the drug at various time

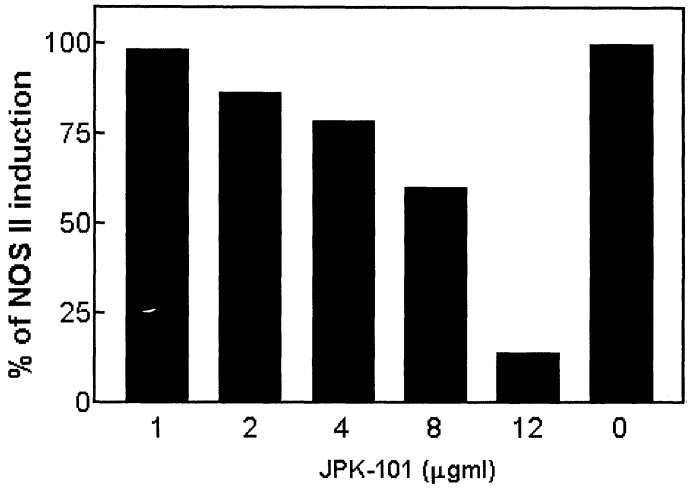
**Figure 6.3: Effects of TwHf compounds on NOS II induction by LPS-stimulated J774A.1**

The cells, obtained from the experiment described in legends to Figure 6.2, were harvested and extracted for total proteins. The lysates were subjected to western blotting analysis with a rabbit polyclonal anti-NOS II antibody, as detailed (Section 2.9.2). Levels of NOS II display were then quantified by densitometry, and normalized against that of the control (J774A.1 treated with only LPS) by Graph Pad Prism software. (**A**) and (**B**), JPK-101; (**C**) and (**D**), JPK-109; and (**E**) and (**F**), JPK-113. The results are expressed as mean ± SD of three independent experiments.

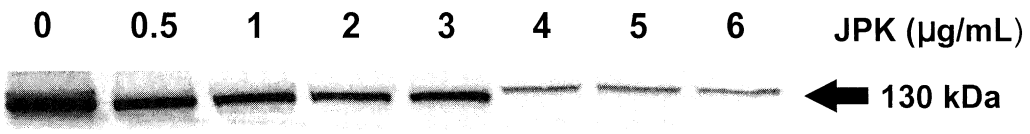
A


0
1
2
4
8
12
JPK (μg/mL)
130 kDa

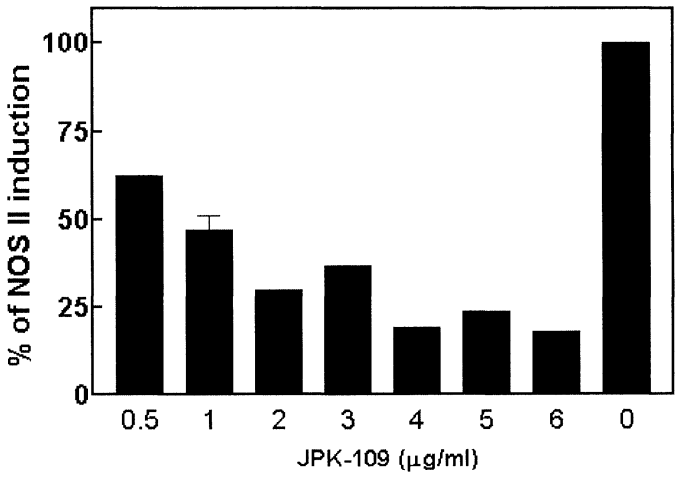
B


% of NOS II induction
100
75
50
25
0
1
2
4
8
12
0
JPK-101 (μgml)

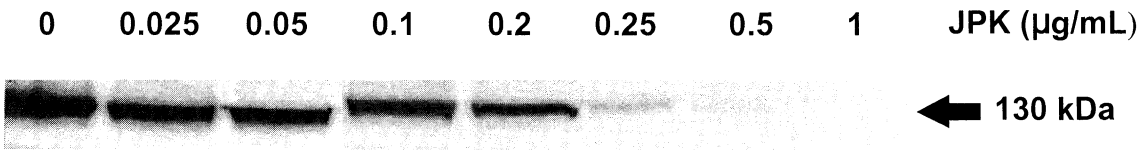
C


0
0.5
1
2
3
4
5
6
JPK (μg/mL)
130 kDa

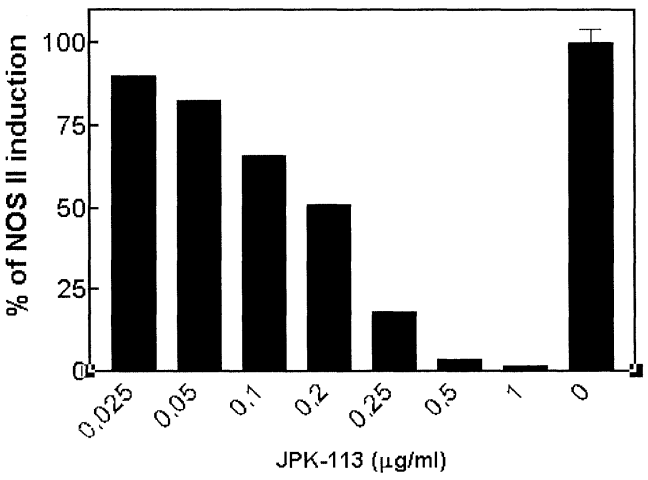
D


% of NOS II induction
100
75
50
25
0
0.5
1
2
3
4
5
6
0
JPK-109 (μg/ml)

E


0
0.025
0.05
0.1
0.2
0.25
0.5
1
JPK (μg/mL)
130 kDa

F


% of NOS II induction
100
75
50
25
0
0.025
0.05
0.1
0.2
0.25
0.5
1
0
JPK-113 (μg/ml)

points before, and where appropriate, after LPS stimulation. Then, we assessed its ability to suppress NOS II expression and NO production in these cells at each of the time points. The objective of the experiment was to delineate the time line when JPK-109 was active. This information may provide a clue as to which of the mechanism(s) mentioned above contributes. As evident in Figure 6.4 A, JPK-109 was very effective at inhibiting NO synthesis, suppressing near complete NO production when given 15 min before or after LPS stimulation. When the cells were treated with JPK-109 1 h after being activated with LPS, their capability to release NO was suppressed by 80-90%. With regards to NOS II expression, the profile was relatively similar to that of NO in that when the drug was added to the cells 15 min after their activation with LPS, it inhibited NOS II induction by almost 100% (Fig. 6.4 B). The presence of the drug at 1 h after LPS stimulation was still able to abrogate the level of NOS II by 80%. The data, although inconclusive at this point, suggests that the drug may exert its action by interfering with the activation of various transcription factors involved in signal transduction of NOS II, which may include, but are not yet proved, AP-1, NF-IL6 and/or NF-κB.

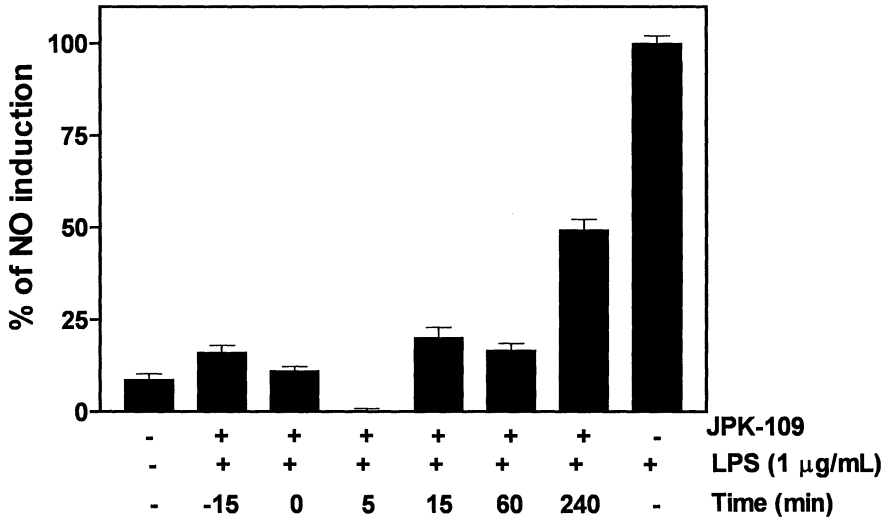
### 6.3.4 Effects of TwHf compounds on TNF-α production by monocytic cells

In addition to examining the modulatory effect of the TwHf compounds on

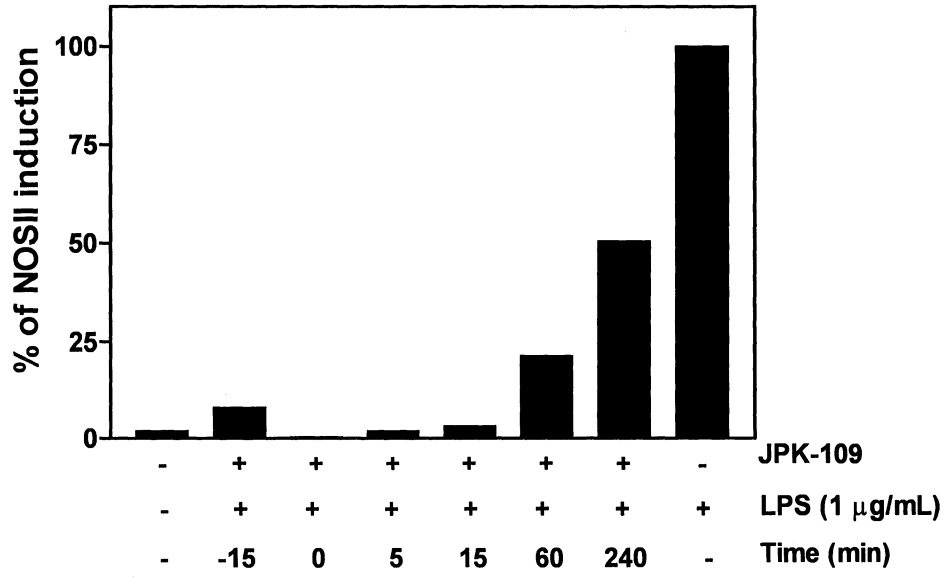
**Figure 6.4: A time course study of LPS-induced NO and NOS II inhibition in J774A.1 cells by JPK-109**

Murine J774A.1 (2.5 x $10^6$ per well) cell was plated in a 6-well plate, and incubated overnight. After which time, medium was removed, and the cells were supplemented with fresh medium containing 1 µg/mL LPS. JPK-109 (3 µg/mL) was added to the cells at different time intervals before or after LPS addition. After an 18-h incubation, cell-free supernatant (100 µL) was measured for NO by the Griess assay while total cell extracts were subjected to western blotting analysis, as described (Section 2.2.11). Levels of NOS II display were quantified by densitometry, and normalized against that of the control (J774A.1 treated with LPS only) by Graph Pad Prism software. (**A**), Inhibition of NO production determined by the Griess assay. (**B**), Inhibition of NOS II expression assessed by western analysis. The results are expressed as mean ± SD of three quadruplicate (for NO measurement), or three single sample experiments (for NOS II investigation).

A


% of NO induction
100
75
50
25
0
- + + + + + + -
- + + + + + + +
- -15 0 5 15 60 240 -
JPK-109
LPS (1 μg/mL)
Time (min)

B

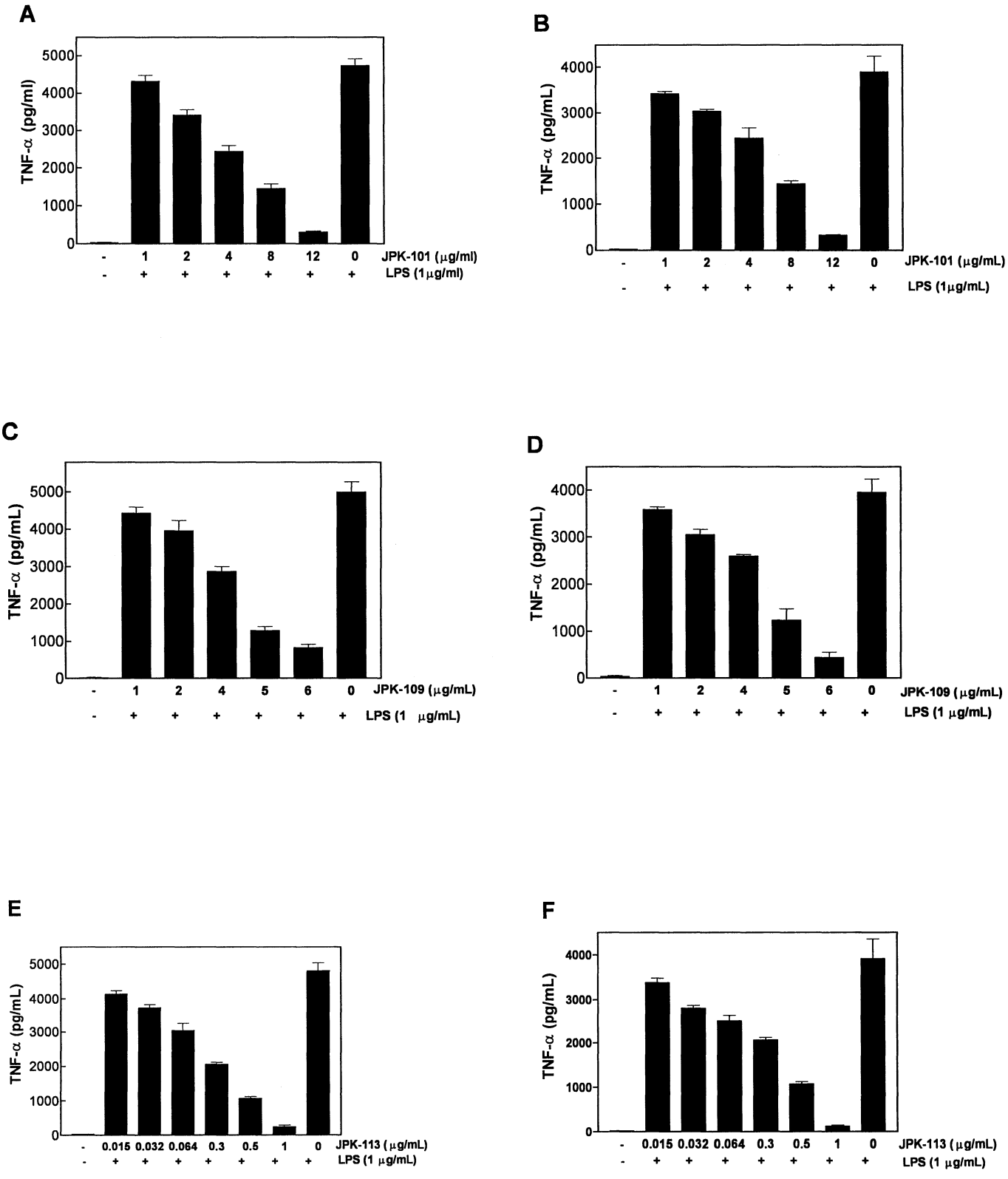

% of NOSII induction
100
75
50
25
0
- + + + + + + -
- + + + + + + +
- -15 0 5 15 60 240 -
JPK-109
LPS (1 μg/mL)
Time (min)

both NOS II induction and NO production by macrophages, we wished to investigate if these three drugs also had any effects on TNF-α release by monocytic cells. To do this, human MM6 and murine J774A.1 monocytic cells were exposed to the compounds and then to LPS, as described above. Cell-free supernatants were evaluated for TNF-α levels by the bioassay with L929 fibroblast cells.

When JPK-101 was screened for its effects on TNF-α production by murine J774A.1 cells, there was a gradual but steady decrease in cytokine release at increasing concentrations of the compound with slight inhibition seen at the lowest dose studied (1 μg/mL). Approximately 50% of TNF-α production was suppressed at approximately 4.0 μg/mL of JPK-101 (Fig. 6.5 A). Although an even more drastic reduction in cytokine release was observed at higher JPK-101 concentrations (at 8 μg/mL and above), the finding may not have much significance physiologically as the $TC_{50}$ for JPK-101 in J774A.1 cells was about 7.2 μg/mL (Fig. 6.1 A). The inhibitory effect of JPK-101 on TNF-α production was also extended to human MM6 cells, albeit less strongly (Fig. 6.4 B). Similar assessment of JPK-109 in the two cell lines showed that in both cases 50% of the inhibition was between 4-5 μg/mL (Fig. 6.5 C and D). JPK-113 (Fig. 6.5 E and F) was not as effective as the others in suppressing TNF-α production in that 50% reduction was not observed in either cell line at doses below the $TC_{50}$ (see Fig. 6.1 E and F).

**Figure 6.5: Effects of TwHf compounds on LPS-mediated TNF-α production by monocytic cells**

(**A**), (**C**), and (**E**), human MM6 and (**B**), (**D**), and (**F**), murine J774A.1 cells ($2.5 \times 10^6$ per well) were seeded in a 6-well plate and incubated overnight. The next morning, old media was removed, and cells supplemented with new media containing appropriate TwHf compounds. 45 min later, LPS (1 μg/mL) was added, and cells cultured for 24 h. Cell-free supernatants (50 μL) were measured for TNF-α by the L929 fibroblast cytotoxicity bioassay (Section 2.2.13). Untreated or LPS (only)-treated cells served as negative and positive controls, respectively. TNF-α production in cells treated with the TwHf compound was normalized against that produced by cells treated with LPS alone. Inhibition of LPS-induced TNF-α production by MM6 and J774A.1 cells exposed to (**A**) and (**B**), JPK-101; (**C**) and (**D**), JPK-109; and (**E**) and (**F**), JPK-113. The results were analyzed by Graph pad Prism, and expressed as mean ± SD of three quadruplicate experiments.


A
TNF-α (pg/ml)
5000
4000
3000
2000
1000
0
- 1 2 4 8 12 0 JPK-101 (μg/ml)
- + + + + + + LPS (1μg/ml)
B
TNF-α (pg/mL)
4000
3000
2000
1000
0
- 1 2 4 8 12 0 JPK-101 (μg/mL)
- + + + + + + LPS (1μg/mL)
C
TNF-α (pg/mL)
5000
4000
3000
2000
1000
0
- 1 2 4 5 6 0 JPK-109 (μg/mL)
- + + + + + + LPS (1 μg/mL)
D
TNF-α (pg/mL)
4000
3000
2000
1000
0
- 1 2 4 5 6 0 JPK-109 (μg/mL)
- + + + + + + LPS (1 μg/mL)
E
TNF-α (pg/mL)
5000
4000
3000
2000
1000
0
- 0.015 0.032 0.064 0.3 0.5 1 0 JPK-113 (μg/mL)
- + + + + + + + LPS (1 μg/mL)
F
TNF-α (pg/mL)
4000
3000
2000
1000
0
- 0.015 0.032 0.064 0.3 0.5 1 0 JPK-113 (μg/mL)
- + + + + + + + LPS (1 μg/mL)

### 6.3.5 Investigation of effect of JPK-101 on MMP production by LPS-stimulated MM6 cells

Although inhibitory effects of TwHf extracts on MMP production by chondrocytes have been demonstrated (Sylvester *et al.*, 2001), their effects on the synthesis of MMP by monocytic cells were unknown prior to this study. To look into this possibility, we studied the effects of JPK-101 on MMP in human MM6 cells before and after LPS activation. As with previous experiments, MM6 cells were treated with various concentrations of JPK-101 for 45 min and then with LPS. After 24 h, total cell lysates were analyzed for expression of MMP by zymography (Section 2.2.18). As illustrated in Figure 6.6, JPK-101 did not appear to significantly cause any changes in the induction of MMP-2 and MMP-9 by MM6 cells before or following their exposure to LPS.

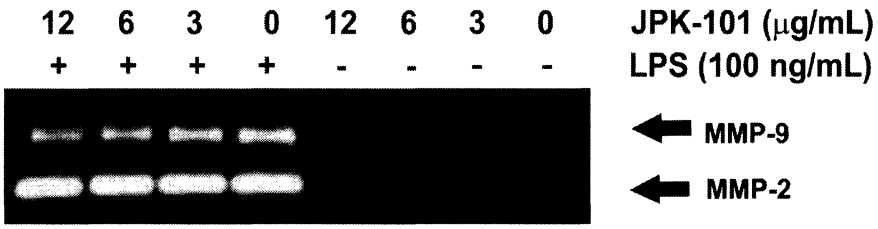
## 6.4 SUMMARY AND DISCUSSIONS

In the present investigation, we evaluated effects of one natural (JPK-113) and two synthetic (JPK-101 and JPK-109) TwHf compounds on macrophage functions by assessing their ability to either stimulate or suppress NOS II expression/NO production, TNF-α release, and MMP induction. Preliminary experiments were also carried out to elucidate mechanism(s) of actions for the compounds.

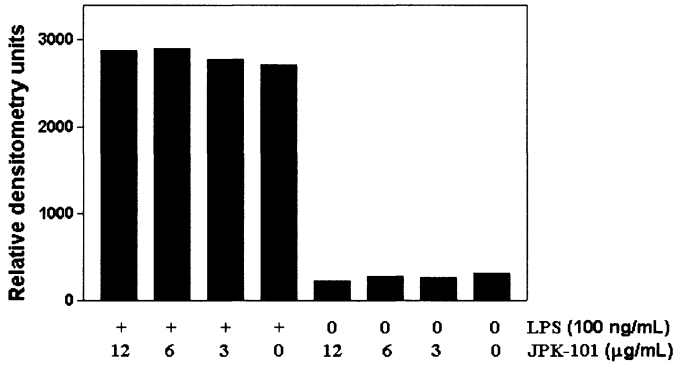
**Figure 6.6: Effect of JPK-101 on MMP production by LPS-stimulated monocytic cells**

MM6 cells ($10^6$ per well) were treated with varying concentrations of JPK-101 for 45 min and then with LPS (100 ng/mL) for 24 h. Total cell extracts were prepared, and the proteins were subjected to zymography analysis, as detailed (Section 2.2.8). Levels of enzyme expression was subsequently quantified by densitometry (Section 2.2.21). (**A**), A zymogram of MMP-2 and MMP-9 gelatinases. Lane 1, untreated MM6 cells; lane 2, 3 µg/ml JPK-101; lane 3, 6 µg/ml JPK-101; lane 4, 12 µg/ml JPK-101; lane 5, LPS; lane 6, LPS + 3 µg/ml JPK-101; lane 7, LPS + 6 µg/ml JPK-101; lane 8, LPS + 12 µg/ml JPK-101. (**B**), MMP-2, and (**C**), MMP-9 expression was quantified by a Chemi Imager (Section 2.2.21).

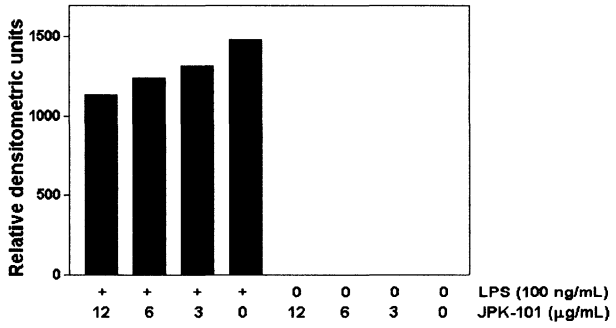
A


12 6 3 0 12 6 3 0
JPK-101 (μg/mL)
+ + + + - - - -
LPS (100 ng/mL)
MMP-9
MMP-2

B


Relative densitometry units
3000
2000
1000
0
+ + + + 0 0 0 0 LPS (100 ng/mL)
12 6 3 0 12 6 3 0 JPK-101 (μg/mL)

C


Relative densitometric units
1500
1000
500
0
+ + + + 0 0 0 0 LPS (100 ng/mL)
12 6 3 0 12 6 3 0 JPK-101 (μg/mL)

With respect to induction of NOS II and production of NO, we found that the three compounds were all effective in attenuating both NOS II display and NO release in a dose-dependent manner, albeit at different dosage. The TI value, a measure for therapeutic efficacy, for NO is 3.32 for JPK-101, 5.82 for JPK-109, and 2.26 for JPK-113 suggesting that all three are potentially useful in the development of therapeutic approaches. It should be noted that the TI values for NO for these compounds in murine J774A.1 cells are lower than those previously found in our laboratory (Barrett, 1998) because of the higher $TC_{50}$ value. A possible explanation for the discrepancy is probably due to differences in the cells used in this current work, which made them more susceptible to the drug. Such differences may also lead to possible alteration in function of the succinate-tetrazolium reductase system of the mitochondrial respiratory chain, the key component of the MTT assay (Section 2.2.7), which in turn would lead to differences in the MTT reading.

With regards to the mechanism of action (s) of these compounds, a time-course study was conducted for JPK-109, and the data showed that this compound exhibited near complete suppression of NOS II/NO 15 min after cell activation with LPS and approximately 80% inhibition of NOS II induction 1 h after LPS stimulation. In addition, the compound remained effective even added 4 h after the cells had been treated with LPS. Taken together, the results suggest that JPK-109 inhibits NO production not by interfering with the LPS receptor binding and early signalling events from the receptor, which usually occurs, respectively, at approximately 5 min

and 15-20 min. Rather, JPK-109 perhaps targets signalling molecules further downstream to prevent activation of various transcription factors (*e.g.,* AP-1 and NF-κB) involved in the signalling pathway leading to NOS II induction (Sylvester *et al.*, 2001). Obviously, some of these transcription factors are shared by many different genes including those for chemokines such as RANTES/CCL5 and inflammatory cytokines such as IL-1. However, at this point we can only offer these possibilities as we do not have any concrete evidence to eliminate one or another. Further experimentation is needed: (1) to fully understand the precise mechanism of JPK-109 action, and (2) to determine which might be the target(s) for this chemical compound. Such understanding may also provide important insights into the mode of action for JPK-101 and JPK-113.

In addition to suppressing NO synthesis and NOS II induction, our studies have also shown that all three compounds could inhibit the release of the pro-inflammatory cytokine TNF-α by both human and murine monocytic cells following their activation with LPS. This effect of the three TwHf compounds is in agreement with their use in traditional medicine as anti-inflammatory mediators. Taken together, the data obtained from our experiments have provided important insights on monocyte functions. First, these TwHf compounds have no "default" effect on naive cells but can significantly suppress induction of LPS-induced NOS II as well as production of NO and TNF-α reflecting their known anti-inflammatory activity. In contrast, JPK-109 was not found to have any inhibitory effect on MMP expression

in human LPS-activated monocytic cells suggesting that the LPS-triggered signalling pathways leading to MMP expression and to production of NO and TNF-α are distinct. It will be of great interest to investigate other immunoregulatory activities of these compounds and possibly piece together the puzzle of how the extracts from TwHf has such broad activity against many different inflammatory diseases (Section 6.1.2).

# CHAPTER SEVEN

# INVESTIGATION OF THE EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE AND PKC-ETA EXPRESSION IN MONOCYTE-DERIVED MACROPHAGES FROM PATIENTS WITH INFLAMMATORY DISEASES

## 7.1 INTRODUCTION

As discussed in Chapter 3 (Section 3.1.1), NOS II expression is only induced by LPS or cytokines such as TNF-α and INF-γ as a result of infection or inflammatory diseases. When the presence of NO is no longer needed, the expression of NOS II enzyme will be shut off, or NOS II-expressing cells eliminated by apoptosis. Failure to do so can result in tissue damage to the host via mechanisms discussed earlier (Section 3.1.3). In fact, it has been shown in a number of studies that NO is associated with pathogenesis of a number of inflammatory conditions, including IDDM (Suarez-Pinzon *et al.*, 2001), AIDS-associated dementia (Adamson *et al.*, 1999; Vincent *et al.*, 1999), and RA (St. Clair *et al.*, 1996; Mapp *et al.*, 2001).

Peroxynitrite, formed from the combination of NO and superoxide, has been shown to be in great abundance in islet beta cells of non-obese diabetic mice (NOD) (Suarez-Pinzon *et al.*, 2001). However, in mice treated with guanidineoethyl-disulphide, a selective NOS II inhibitor, the proportion of the beta-islet cells that were positive for peroxynitrite became significantly decreased after the treatment (Suarez-Pinzon *et al.*, 2001). In other studies, cultures of isolated beta-islets revealed resident macrophages to be a major source of IL-1, which lead to upregulation of NOS II expression and NO production. A consequence of this was an increase in beta cell death by apoptosis (Hirasawa *et al.*, 1997; Reddy and Young, 2002).

Our investigation of PKC expression in monocytic cells (Chapter 5) revealed that PKC-η was completely deficient in the human cells and that NOS II could not be induced in human mononuclear phagocytes following exposure to LPS. Subsequent transfection studies of human monocytic MM6 cells with PKC-η resulted in NOS II expression and NO production following endotoxin stimulation (Chapter 5). Therefore, these data suggested that PKC-η expression may be required prior to the induction of the NOS II gene in human monocytic cells. In light of these findings, we set out to further test this hypothesis to determine whether we could establish a correlation between PKC-η and NOS II in clinical situations. We chose to conduct the investigation in AIDS/HIV-infected patients. The choice stemmed from earlier findings connecting elevated NO levels and the inflammatory response seen in patients with this disease (Torre *et al.*, 1996). It was shown that elevation in NO was evident, especially in AIDS/HIV-infected patients succumbed to opportunistic infections (Torre *et al.*, 1996). Although we subsequently found an increase in plasma NO from AIDS/HIV-infected patients (Section 7.2.1 below), the work could not be continued due to great limitation in obtaining a sufficient number of peripheral blood monocytes from these patients. Therefore, we pursued further investigation with samples from arthritis patients. This model was plausible because of findings from several studies demonstrating upregulation of NO production in inflammatory arthritis patients (Mapp *et al.*, 2001; St-Clare *et al.*, 1995). For example, St-Clare *et al.* (1995) reported elevation of NOS II expression

in PBMC in RA patients. Mapp *et al.* (2001) showed that 3-nitrotyrosine, a footprint marker of peroxynitrite, along with other NO-derived intermediates, was found in macrophages in the inflamed synovium. Also, elevated NO levels have been associated with an increase in apoptosis of the cells lining the synovial layer and articular cartilage (van't Hof *et al.*, 2000). Our decision to study this disease was strengthened by findings from studies in animal models which supported an association between NO and joint swelling/destruction in arthritis. In these investigations, the NOS inhibitor $HN^G$-monomethyl L-arginine monoacetate was shown to lead to amelioration of adjuvant-induced (Stefanovic-Racic *et al.*, 1995) and virus-induced (McCartney-Francis *et al.*, 2001) arthritis.

RA is one of the most common human autoimmune diseases characterized by inflammation of the joints (prevalence of 1%). The principal pathological features of joint inflammation include invasion of leukocytes, their proliferation in the synovium and accumulation of peripheral mononuclear cells in the synovial fluids. It is thought that infiltration of these cells results in overproduction of TNF-$\alpha$ and IL-1, as well as other inflammatory mediators including NO, MMP, and PGE2 (Abramson *et al.,* 2001). Dysregulation of these molecules eventually leads to damage to the joints. In some cases, extra articular disease may be manifested, and survival impaired.

The spondyloarthropathies (SpA) are a group of inflammatory joint diseases characterized by an involvement of both synovium and entheses leading to spinal

and peripheral arthritis (Keat and Arnett, 2001). Although they were once considered as "variants" of RA, SpA are now known to be entities distinct from RA for three major reasons. First, SpA primarily involve axial inflammation and to a lesser extent, articular and extra-articular manifestations. Second, SpA patients are generally serologically negative for the rheumatoid factor (RF). Third, in most cases there is a strong association with MHC class I, HLA-B27. Ankylosing spondylitis (AS), psoriatic arthritis (PsA), Crohn's, and Reiters are the principal clinical entities of SpA. AS is an inflammatory arthropathy that preferentially targets the axial skeleton, and has a male prevalence of 3:1. Systemic manifestations of this disease occur in one third of the patients. It is estimated that nearly 30% of patients with psoriasis, with the disease itself having a prevalence of 1-3% adults, eventually develop PsA (Smiley, 1995).

Majority of the data presented and discussed in this chapter have been published in the *Journal of Rheumatology* (Pham *et al.*, 2003b).

## 7.2 PATIENTS

Blood samples were obtained after written consent had been given, and the project was approved by the Human Investigation Committee (Memorial University). Laboratory investigators were blind to the diagnosis until completion of all analyses.

### 7.2.1 AIDS and HIV-infected

Serum samples from thirteen patients with AIDS or infected with HIV were used in the evaluation of NO levels, and compared with values from a control group (see below).

### 7.2.2 Inflammatory arthritides (IA)

Twenty patients (12 females and 8 males, aged 24-76), who fulfilled the American College of Rheumatology Criteria (Arnett *et al.*, 1988) were recruited for this study. Fourteen of these individuals (aged 36 to 76) had RA, while the other six had SpA (peripheral synovitis, aged 24 to 63). The SpA group was made up of three patients with AS and three with PsA. Based on clinical assessments of the number of swollen/tender joints at the time of study, patients with RA (RF-positive) and PsA (RF- negative) were further categorized as having severe (>10 active joints) (active disease), or mild inflammation (< 5 active joints). The patients with AS were classified as being either clinically quiescent or having inflammation.

One patient in the inflammatory arthritic group did not receive any treatment (5%). 70% of the patients received Methotrexate (MTX), and 55% were on steroids (Table 7.1).

### 7.2.3 Control groups

For the evaluation of plasma NO in patients afflicted with AIDS or HIV infection, plasma samples from 14 healthy volunteers were obtained, and analyzed in the same way as the patient samples. For the study with IA patients, nine healthy volunteers (9 females, aged 22-48) and thirteen patients (8 females and 5 males, aged 47 to 81) with osteoarthritis (OA), who fulfilled the American College of Rheumatology Criteria for OA, were recruited. Most, if not all OA patients, received only non-steroidal anti-inflammatory drugs (NSAIDS, *e.g.*, rofecoxib).

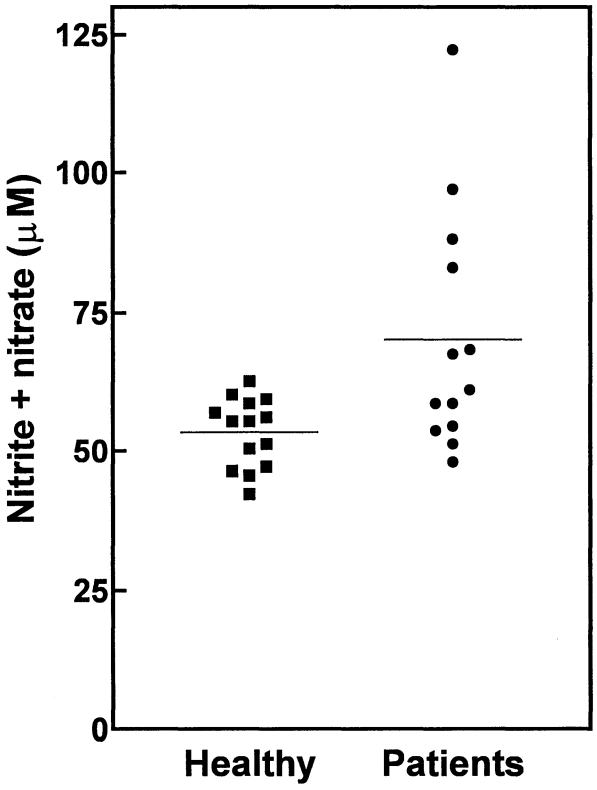
## 7.3 RESULTS

### 7.3.1 Assessment of NO in the plasma of HIV-infected patients

In light of findings suggesting apparent involvement of NO in the inflammatory process associated with AIDS and its related disorders (Adamson *et al.*, 1999; Vincent *et al.*, 1999), we evaluated the levels of NO in serum samples from thirteen AIDS or HIV-infected patients by Griess assay. As shown in Figure 7.1, AIDS patients or those infected with HIV, as a group, had elevated NO levels (70.1 ± 21.7 μM) in the circulation compared to the healthy control group (53.4 ± 6.2 μM). However, it should be noted that there is a weak difference in NO levels among the patients, as depicted by the high standard deviation value (21.7 μM). Many of these patients had circulating NO within the normal range, but four of them

## Figure 7.1: Assessment of plasma NO in patients with AIDS/HIV-infection

NO was measured in 50 µL of plasma from healthy volunteers (n = 14) or patients with AIDS/HIV-infection (n = 13). Nitrate was first reduced by nitrate reductase to nitrite, which was then measured by the Griess assay (Section 2.2.12.2). Statistical analysis with unpaired Student's *t*-tests showed a significant elevated plasma NO in the patients (70.2 ± 21.7 µM) when compared to that in the healthy subjects (53.42 ± 6.3 µM). The *p* from the analysis was 0.0165.


125
100
75
50
25
0
Nitrite + nitrate (μM)
Healthy
Patients

had a much higher NO value.

### 7.3.2 Investigation of plasma NO in IA patients

NO levels in plasma from patients with IA (RA, n = 14 and SpA, n = 6), OA (n = 13), and healthy individuals (n = 9) (Fig. 7.2) were assessed by measurement of its stable products nitrite and nitrate using the Griess assay. As evident, all ten IA patients (8 RA and 1 SpA) with severe inflammation/active disease (Fig. 7.2, severe SpA and RA) had highly elevated NO (237.8 ± 34.4 μM) relative to that seen in the 13 patients with OA (126.9 ± 40.9 μM) (Fig. 7.2, OA), or in healthy individuals (131 ± 18.9 μM) (Fig. 7.2, healthy). Similarly, ten IA patients (6 RA and 4 SpA) with mild inflammation (<5 swollen joints) had slightly higher plasma NO (145.7 ± 20.2 μM) as that in the two control groups (OA and healthy). This elevation was not statistically significant by unpaired two-tailed Student's *t*-tests ($p$=0.11 and $p$= 0.53, respectively). It is clear from the data that the levels of NO in the circulation are strongly correlated with the degree of joint inflammation, as the severely inflamed had higher plasma NO than the mildly inflamed, who in turn had higher levels than osteoarthritis. It should be noted that the plasma NO previously observed in the healthy control individuals tested in parallel with the HIV-infected/AIDS patients (Fig. 7.1) was lower than that for the healthy group used as a control group in the study of IA patients. The discrepancy is likely due to different experimental conditions. During the course of the investigation (from HIV-infected/AIDS to IA

**Figure 7.2: Elevated plasma NO in IA patients**

NO was measured from 50 µL of plasma from healthy volunteers (n = 9), RA (n = 14), SpA (n = 6), and OA (n = 13). Nitrate was first reduced to nitrite by nitrate reductase and the total nitrite was then quantified by the Griess assay. SpA patients are inflammatory arthritides, who are serologically RF-negative of "severe" (minimum of 5 swollen joints) or "mild" (< 5 swollen joints) inflammation. Each dot represents a patient and the mean (± SD) indicated.

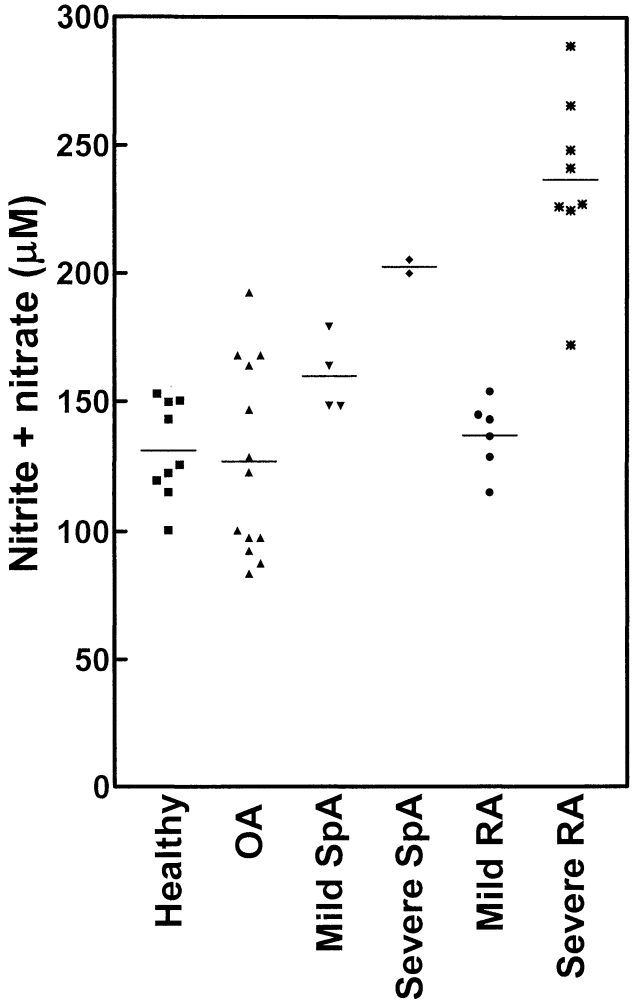
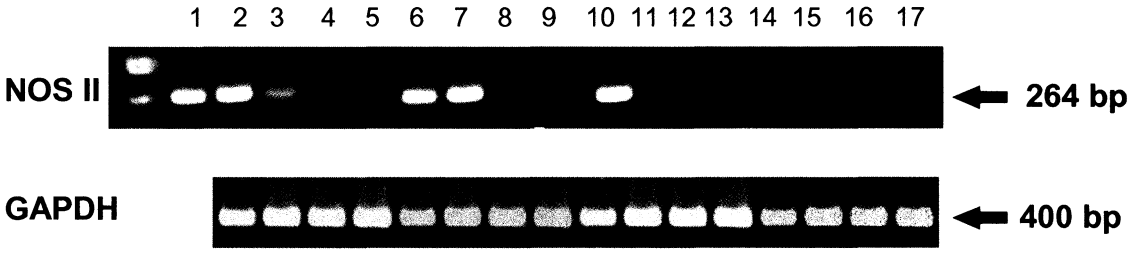

Nitrite + nitrate (μM)
300
250
200
150
100
50
0
Healthy
OA
Mild SpA
Severe SpA
Mild RA
Severe RA

**Figure 7.3: NOS II expression in MDM from IA patients with severe inflammation**

MDM from healthy individuals and arthritis patients were isolated, cultured and assessed for NOS II expression by RT-PCR using gene specific primers. GAPDH amplified from the same samples was used to control for sample loading. PCR products (9 µL) were separated on a 1% EtBr-containing agarose gel, visualized by a UV light source, and the bands digitally photographed. 0.5 µg of 100 bp DNA ladder was loaded as molecular markers. Lane 1, recombinant NOS II fragment (excised from pBABE) served as a positive control; lane 2, case 42 (PsA, severe); lane 3, case 39 (AS, inflammation); lane 4, case 38 (AS, clinically quiescent); lane 5, case 37 (AS, clinically quiescent); lane 6, case 29 (RA, severe); lane 7, case 28 (RA, severe); lane 8, case 26 (RA, mild); lane 9, case 24 (RA, mild); lane 10, case 23 (RA, severe); lane 11, case 22 (OA, no inflammation); lane 12, case 18 (OA, no inflammation); lane 13, case 14 (OA, no inflammation); lane 14, case 11 (OA, no inflammation); lane 15, case 4 (healthy); lane 16, case 2 (heathy), and lane 17, case 1 (healthy).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
NOS II
264 bp
GAPDH
400 bp

patients), the assay was modified in a hope to achieve the optimal conditions for the conversion of nitrate to nitrite by nitrate reductase. In the end, parameters such as longer incubation time and higher enzyme concentrations were adopted.

### 7.3.3 Association between plasma NO, NOS II, and PKC-η in peripheral blood monocyte-derived macrophages

In Chapter 5, we showed that only after human monocytic MM6 cells were transfected with PKC-η were they capable of expressing NOS II and releasing NO in the culture media following LPS stimulation. From these data we proposed that PKC-η expression had to precede/or accompany NOS II expression in human monocytic cells, at least in the cell line model we studied. Therefore in this chapter, we wished to test this hypothesis to see if this could also be applicable to *in vivo* situations.

In Figure 7.2, we showed that plasma NO levels were significantly elevated in RA patients with active disease, and that the absolute levels were correlated with progression of the conditions. Subsequently in Figure 7.3, we showed that RA and SpA patients with active disease (cases 23, 28, 29, and 42; Table 7.1) strongly expressed NOS II mRNA, but healthy individuals (cases 1, 2, and 4) or those with OA (cases 11, 14, 18, and 22; Table 7.1) did not. Also, MDM cells from IA patients with normal circulating NO did not express NOS II (cases 24, 26, and 38; Table 7.1). The findings, thus, suggest that the elevation of plasma NO was associated with

NOS II expression in MDM from these patients. Therefore, in the next step, we wanted to determine if there was any correlation in expression between NOS II and PKC-η in MDM from these patients. To do this, we screened for the presence of PKC-η in MDM by RT-PCR. Figure 7.4 represents data from one such experiment. PKC-η was found to be highly expressed in RA patients with active disease (cases 23, 28, 29, and 42; Table 7.1). In sharp contrast, healthy volunteers (cases 2 and 4) and OA patients (cases 14, 18 and 22), who lacked NOS II, were also negative for PKC-η. Also, as seen in the cases of NOS II, IA patients who did not have active disease, and who had normal circulating NO did not express PKC-η (cases 26, 37, and 38). Taken together, only in IA patients with severely active disease did we observe: (1) a statistically significant ($p < 0.0021$) up-regulation of NO in plasma compared to that seen in the control healthy individuals, and patients with OA, or with mildly inflammatory RA (Fig. 7.2) and (2) a constitutive co-expression of PKC-η and NOS II in MDM from severely affected IA patients. These data were fully summarized in Table 7.1.

Interestingly, one anomaly to our hypothesis was observed in Patient 23 (Table 7.1). Clinical assessments of the number of tender and swollen joints had shown that this particular individual had severe inflammation with more than 10 active joints. Plasma NO level in this patient (227 μM) was notably elevated when compared to that of the control individuals. In accord with this finding, RT-PCR analysis showed the expression of NOS II in MDM. However, PKC-η appeared to

**Figure 7.4: Display of PKC-η mRNA in MDM from IA patients with severe inflammation**

MDM from healthy individuals and arthritis patients were isolated, cultured and assessed for PKC-η expression by RT-PCR using gene specific primers. GAPDH amplified from the same was used as a loading control. PCR products (9 µL) were separated on a 1% EtBr-containing agarose gel, visualized by a UV light source, and digitally photographed. 100 bp DNA ladder (0.5 µg) was loaded as molecular markers. Lane 1, no-template PCR (negative control); lane 2, case 42 (PSA, severe); lane 3, case 39 (AS, inflammation); lane 4, case 38 (AS, clinically quiescent); lane 5, case 37 (AS, clinically quiescent); lane 6, case 29 (RA, severe); lane 7, case 28 (RA, severe); lane 8, case 26 (RA, mild); lane 9, case 25 (RA, severe); lane 10, case 24 (RA, mild); lane 11, case 23 (RA, severe); lane 12, recombinant PKC-η fragment (excised from pks1.PKC-η plasmid); lane 13, case 22 (OA, no inflammation); lane 14, case 18 (OA, no inflammation); lane 15, case 14 (OA, no inflammation); lane 16, case 4 (healthy), and lane 17, case 2 (healthy).

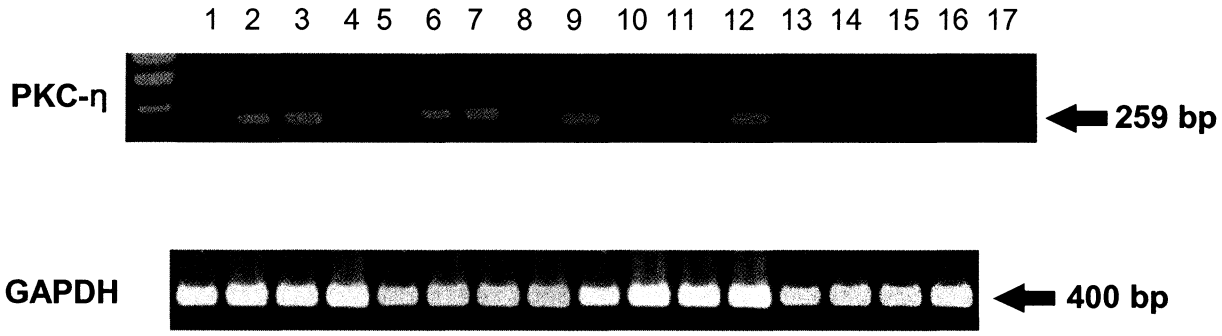

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
PKC-η
259 bp
GAPDH
400 bp

**Table 7.1: mRNA co-expression of NOS II and PKC-η is correlated with elevated plasma NO in IA patients**

F, female; M, male; N/A, not applicable; DMARD, disease modifying anti-rheumatic drug; N, no; Y, yes; N/D, not determined; OA, osteoarthritis; RA, rheumatoid arthritis; MTX, methotrexate; SpA, peripheral spondyloarthropathies; AS, ankylosing spondylitis; PsA, psoriatic arthritis.

***Table 7.1 : Transcriptional co-expression of NOS II and PKC- η with elevated plasma NO in patients with inflammatory arthritis***

| Subject (ID #) | AGE | SEX | $D_x$ | Activity | Steroids | DMARD | NO (μM) | PKC-η | NOS II |
|---|---|---|---|---|---|---|---|---|---|
| 1 (001) | 26 | F | Healthy | N/A | N | N | 143.2 | N | N |
| 2 (002) | 42 | F | Healthy | N/A | N | N | 150.4 | N | N |
| 3 (046) | 22 | F | Healthy | N/A | N | N | 153.0 | N/D | N/D |
| 4 (047) | 25 | F | Healthy | N/A | N | N | 149.8 | N | N |
| 5 (048) | 32 | F | Healthy | N/A | N | N | 122.4 | N/D | N/D |
| 6 (057) | 40 | F | Healthy | N/A | N | N | 119.6 | N | N |
| 7 (058) | 44 | F | Healthy | N/A | N | N | 125.6 | N | N |
| 8 (061) | 48 | F | Healthy | N/A | N | N | 100.5 | N | N |
| 9 (062) | 43 | F | Healthy | N/A | N | N | 115.0 | N | N |
| 10 (006) | 54 | F | OA | Not inflamed | N | N | 146.8 | N | N |
| 11 (007) | 76 | M | OA | Not inflamed | N | N | 168.0 | N | N |
| 12 (009) | 58 | F | OA | Not inflamed | N | N | 168.0 | N | N |
| 13 (020) | 81 | F | OA | Not inflamed | N | N | 192.4 | N/D | N/D |
| 14 (025) | 49 | F | OA | Not inflamed | N | N | 97.6 | N | N |
| 15 (027) | 56 | M | OA | Not inflamed | N | N | 83.6 | N | N |
| 16 (028) | 60 | M | OA | Not inflamed | N | N | 164.0 | N/D | N/D |
| 17 (029) | 61 | F | OA | Not inflamed | N | N | 97.6 | N | N |
| 18 (032) | 47 | F | OA | Not inflamed | N | N | 122.8 | N | N |
| 19 (034) | 48 | F | OA | Not inflamed | N | N | 87.6 | N | N |
| 20 (035) | 55 | F | OA | Not inflamed | N | N | 128.4 | N | N |
| 21 (037) | 50 | M | OA | Not inflamed | N | N | 92.4 | N | N |
| 22 (038) | 63 | M | OA | Not inflamed | N | MTX | 100.4 | N | N |
| 23 (004) | 57 | F | RA | Severe | Prednisone | MTX | 227.2 | N | Y |
| 24 (005) | 39 | F | RA | Mild | Prednisone | MTX | 154.0 | N | N |
| 25 (017) | 36 | M | RA | Severe | N | N | 289.2 | Y | Y |
| 26 (019) | 44 | M | RA | Mild | N | MTX | 143.2 | N | N |
| 27 (021) | 76 | F | RA | Severe | Prednisone | N | 172.2 | N/D | N/D |
| 28 (023) | 54 | M | RA | Severe | N | MTX | 248.4 | Y | Y |
| 29 (030) | 46 | F | RA | Severe | N | MTX | 226.2 | Y | Y |
| 30 (031) | 51 | F | RA | Mild | Prednisone | MTX | 136.6 | N/D | N/D |
| 31 (053) | 50 | F | RA | Severe | N | MTX | 265.7 | Y | Y |
| 32 (054) | 54 | F | RA | Severe | Prednisone | N | 241.3 | Y | Y |
| 33 (055) | 60 | M | RA | Mild | N | MTX | 115.0 | N | N |
| 34 (056) | 52 | F | RA | Severe | Prednisone | MTX | 224.7 | Y | Y |
| 35 (059) | 59 | F | RA | Mild | Prednisone | MTX | 145.0 | N | N |
| 36 (060) | 75 | F | RA | Mild | Prednisone | MTX | 128.6 | N | N |
| 37 (011) | 60 | M | SpA (AS) | Clinically quiet | Prednisone | N | 148.4 | N | N |
| 38 (012) | 24 | M | SpA (AS) | Clinically quiet | N | MTX | 148.4 | N | N |
| 39 (013) | 36 | M | SpA (AS) | Inflamed | Prednisone | N | 205.6 | Y | Y |
| 40 (018) | 37 | M | SpA (PsA) | Mild | N | MTX | 164.0 | N/D | N/D |
| 41 (022) | 33 | F | SpA (PsA) | Mild | N | MTX | 179.3 | N/D | N/D |
| 42 (026) | 63 | F | SpA (PsA) | Severe | Prednisone | N | 200.0 | Y | Y |

N/A, not applicable; N/D, not done.

be completely absent from these cells. Subsequently, we repeated the experiments for this particular case and the results remained the same as that we had originally obtained. At the present time, we have no clear-cut explanation as to why this was the case. However, there is some evidence in the literature suggesting that in some patients with inflammatory diseases who receive a very high dose of steroids, especially prednisone, in combination with disease-modifying antirheumatic drugs such as methotrexate, there is an increased total PKC activity relative to those not receiving this kind of combination treatment. This would, therefore, imply that the increased in total activity of the kinase would lead to a higher turn-over of total PKC expression. This observation may offer a plausible explanation for the absence of PKC-η in macrophages from this particular patient. Having said that, it is prudent to investigate more patients with similar treatment to determine whether this is indeed the case.

## 7.4 SUMMARY AND DISCUSSIONS

Here, we have shown that on average, HIV-infected/AIDS patients have elevated circulating NO compared to that of healthy controls, with some patients displaying significantly higher levels of NO than normal. However, because of the large standard deviations in the NO values between the different patients relative to that seen in the healthy group, it is rather difficult to evaluate any significance of the elevation of NO with respect to its pathophysiology in rheumatic diseases.

Unfortunately, it was not possible for us to continue with this work due to the unavailability of the material.

The elevation of NO was very pronounced in the circulation of IA patients with severe disease. Specifically, the data showed that the severely affected individuals had significantly elevated serum NO compared to OA patients. The latter, in turn, had comparable NO levels to healthy individuals (Fig. 7.2). These elevations in NO levels appeared to correlate with circulating monocyte expression of the NOS II gene as its mRNA accumulation was also increased in these same patients (Fig. 7.3 and Table 7.1). Although up-regulation of NO in plasma of individuals with RA has been reported previously (Ueki *et al.*, 1996), our findings are novel from two aspects. First, we were able to show that the elevated NO levels were only observed in patients with active disease, which is defined by a tender joint score of ten or greater. IA patients, with mild inflammation, had comparable levels of NO to those found in healthy people or in OA patients. Second, to the best of our knowledge, this is the first time that a positive correlation between PKC-η and NOS II expression by MDM has been reported in arthritic patients. Specifically, the two genes were either both expressed or not expressed at all. Our hypothesis proposing that PKC-η expression is required prior to or at the same time as NOS II was supported by the findings of all but one cases studied. The results from case 23 contradicted our hypothesis in that this individual with severely active RA showed elevation in plasma NO and NOS II expression in peripheral MDM.

However, in contrast to the other patients in the same category, who also had similar active disease and were double positive for the two genes, this patient did not appear to express PKC-η in MDM.

Although TNF-α and IL-1 have long been considered as attractive targets for pharmacologic intervention and gene therapy for inflammatory diseases like arthritis, NOS II has also begun to gain attention as yet another candidate for the development of novel treatments for the disorders. In recent years, many compounds designed by different groups have been used to study their effects on the expression of NOS II. Some of these include: (1) L-arginine derivatives (*e.g.*, $HN^G$-monomethyl L-arginine monoacetate [L-NMMA]) that compete at the active site (Weinberg *et al.*, 1994; Amin *et al.*, 1999), (2) metabolic inhibitors (*e.g.*, citrulline analogues, aminoguanidine) (Salerno *et al.*, 1995), and (3) inhibitors of dimerization, as NOS monomer is inactive (Xie *et al.*, 1996; Ohtsuka *et al.*, 2002). In some of these studies, the NOS inhibitors were shown to have direct anti-inflammatory effects leading to amelioration of adjuvant or virus-induced arthritis. However, as NO does have other physiological roles (endothelium-relaxing factor and neurotransmitter) besides that of an inflammatory mediator, therapeutic approaches with these compounds may have severe side effects as these compounds are likely to suppress the effects of all three NOS. Obviously, the ultimate goal would be to suppress the over-production of NO in arthritis-affected joints and peripheral blood cells without compromising other important functions of

NO.

In light of the fact that PKC plays an essential role in the signal transduction of various cellular processes including gene expression, several reports have surfaced in the literature proposing a role of PKC inhibitors (benzylidene derivative and staurosporine) as a possible anti-rheumatic agent in that they led to attenuation of IL-1α or PMA-induced IL-1β production by synovial cells from RA patients (Watanabe *et al.*, 1993). In addition, PKC inhibitor gold sodium thiomalate (GSTM) employed as a therapeutic agent for RA for many years has been shown by Hashimoto and colleagues (Hashimoto *et al.*, 1994) to suppress mitogen-induced T cell proliferation suggesting a role of PKC in modulation of the adaptive immune response in arthritis. Similarly, in an experimental model of adjuvant-induced arthritis, PKC inhibitors debromo-hymenialdisine (a catalytic site inhibitor) (DiMartino *et al.,* 1995) and Ro 32-0432 (Birchall *et al.*, 1994) have been shown to alleviate rat paw swelling via suppression of the proliferation of autoreactive lymphocytes. However, whether these PKC inhibitors have any effects on mononuclear phagocytes was not known as they were not investigated in these studies.

In this chapter, we have shown that MDM from all but one patient with active inflammatory arthritis (RA and SpA) expressed both NOS II and PKC-η. The cells are indicative of the inflammatory state of these patients, and their presence in the circulation adds to the systemic burden of this disease and should not be left

untreated. Therapeutic treatments, conventional or otherwise, appear to be uneffective in preventing/controlling the activation of these cells. These findings may pave the way for future investigation of PKC-η as a potentially therapeutic target for the treatment of inflammatory conditions such as RA, where overproduction of NO and/or persistent expression of NOS II by peripheral blood monocytic cells have been shown to contribute to disease pathogenesis (Cawston, 2000; Perkins *et al.*, 1998).

# CHAPTER EIGHT

# INVESTIGATION OF THE CORRELATION OF PLASMA NITRIC OXIDE/ MONOCYTE NITRIC OXIDE SYNTHASE II AND CLINICAL SYMPTOMS IN INFLAMMATORY ARTHRITIS PATIENTS RECEIVING CYTOKINE-TARGETED THERAPIES

## 8.1 INTRODUCTION

It is believed that the chronic inflammatory reaction in the joint synovium associated with conditions like RA and PsA is attributed to over-production of pro-inflammatory cytokines such as TNF-α, IL-1, chemokines, MMP, and free radicals like NO. These molecules, especially TNF-α, play an important role in initiating and perpetuating inflammatory and destructive processes within the rheumatoid joint.

*In vivo,* TNF-α is the most rapidly produced pro-inflammatory cytokine, with serum levels detectable in mice as early as 30 min following induction by mitogenic stimuli (Tracey *et al.*, 1986). Perhaps the initial surge of TNF-α comes from cleavage of membrane bound TNF-α on activated macrophages, T cells and neutrophils by TNF converting enzyme TACE/ADAM17 (Black *et al.*, 1997). Subsequent *de novo* synthesis by these cells ensures steady levels of the cytokine. Because of its early release, TNF-α is though to regulate expression of other late cytokines/chemokines, including IL-1, IL-6, RANTES, and IL-8. This notion is supported by studies where addition of anti-TNF antibodies to *in vitro* cultures of a representative population of cells derived from diseased joints led to inhibition of the aforementioned cytokines/chemokines (Brennan *et al.*, 1989; Feldmann *et al.*, 1996). In addition, the apparent link between TNF-α and disease pathogenesis stemmed from its ability to up-regulate intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both of which are involved in

lymphocyte trafficking to inflammatory lesions (Wakefield *et al.*, 1991). Studies such as these, together with the observation of upregulation of TNF/TNFR on synovial tissues from patient with rheumatic disease (Chu *et al.*, 1991) lend support to a pathogenic role of TNF-α in RA; hence, its promise as a therapeutic target candidate for the disease. Since then, various agents targeting either TNF-α (*e.g.,* Remicade™ [infliximab]) or TNF receptor (*e.g.,* Enbrel™ [etanercept]) have been adopted as novel treatments for RA. Currently, etanercept and infliximab, which are to be administered in conjunction with MTX, one of the most commonly used DMARD, have been approved for the treatment of RA. In earlier studies, both drugs were shown to be effective in patients with other rheumatic diseases including AS and PsA (Brandt *et al.*, 2000; Van den Bosch *et al.,* 2001). Infliximab is a chimeric (mouse Fv1, human IgG1) monoclonal antibody which binds to both soluble and membrane forms of TNF-α with high affinity (Ka = $10^{-10}$ M) (Knight *et al.*, 1993). The medication is believed to help by reducing the progression of structural damage and improving physical function.

In addition to TNF-α, IL-1 is also thought to play an important role in pathophysiology of RA by causing cartilage destruction through inducing the loss of proteolgycan and stimulating bone resorption (Patwari *et al.*, 2003; Abramson and Amin, 2002). Results from randomized clinical trial studies have shown that arthritic patients receiving an IL-1 blocker show improvement in daily physical activities and reduction of symptoms including joint inflammation and stiffness when

compared to those receiving only the placebo (Garces, 2001). As a result, anakinra (Kineret™) has been recently added to the list of medications for the management of RA. Anakinra is a recombinant and non-glycosylated form of the IL-1Ra that directly interferes with the binding of IL-1 to the receptor. Both anakinra and infliximab are often prescribed when the patient has had little or no response to one or more prescribed DMARD.

In rheumatic diseases, several clinical and laboratory parameters are used to assess efficacy of a therapy in patients. Of note, a count of swollen and tender joints and a value of the erythrocyte sedimentation rate (ESR) are usually included in routine assessment of a patient's response to a particular treatment. ESR is a measure of the distance red blood cells (RBC) travel to settle at the bottom of a tube of blood during one hour (Wollheim, 1996). Factors that affect the ESR of RBC include: (1) size of cells; (2) viscosity of plasma, and (3) repellent forces between negatively charged red cell surfaces. The normal range for ESR is 0-15 mm/h in male and 0-20 mm/h in female. The presence of asymmetric proteins, especially fibrinogen and gamma globulins, allows for the formation of rouleaux (cells moving closer together) that cause the cells to settle more rapidly, hence, giving rise to an increased ESR value. Clinically, the ESR is often used as an indirect marker of inflammation, as it is often elevated in inflammatory diseases such as RA. In certain cases, elevation in ESR has been observed in patients with neoplastic diseases (*e.g.*, Hodgkin's, infertvos) (Cawston, 2000).

During the course of our initial investigation for a correlation between plasma NO, PKC-η, and NOS II in IA patients with active disease, we made a novel observation in case 43. Despite experiencing only mild inflammation in the joints, this RA patient still had highly elevated circulating NO (289.2 μM), and expressed both NOS II and PKC-η in MDM. Retroactive consultation with the collaborative rheumatologist led to the discovery that this patient was on infliximab (see above) therapy, which targeted TNF-α function. Therefore, we screened seven more patients to determine if the initial finding with case 43 (see below) was a consequence of a random observation, or may reflect a real trend. This second screening eventually led to another evaluation of NO in plasma and NOS II and PKC-η in MDM from a different group of IA patients. This approach stemmed from the fact that one of the seven patients (case 50, see below) was receiving anakinra instead of infliximab, and the findings from this particular case, warranted further experimentation.

A manuscript containing the data presented in this chapter is to be submitted to a peer-reviewed journal for possible publication.

## 8.2 PATIENTS

A total of fifteen IA patients were studied. Seven of them received infliximab, while the other eight anakinra. Clinical assessments of these patients after the therapy revealed that all of these individuals experienced only mild inflammation,

as determined by the swollen joint count of five or fewer. In addition, in response to the therapy the patients also exhibited reduction in the ESR value.

Plasma and MDM were obtained prepared from each case, as described (Section 2.2.3). Plasma samples was used in assessment of circulating NO levels.

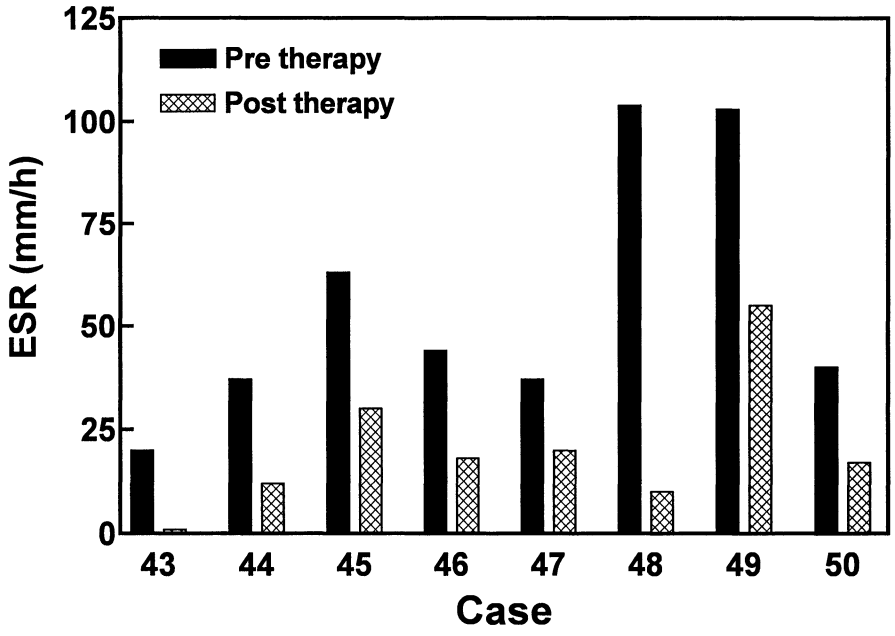
## 8.3 RESULTS

### 8.3.1 Disease activity following therapy with infliximab

Before and after therapy, the patients were assessed for the number of swollen and tender joints as well as the ESR. Figure 8.1 shows that in all eight patients there is a significant reduction in the ESR value after the therapy relative to that before the therapy. It should, however, be noted while the ESR in many of the cases studied did return to normal, that of cases 45 and 49 were still relatively high compared to the normal values (see above). In addition, clinical assessment of the number of swollen and tender (effused) joints from these patients after the therapy and at the time of sample collection showed a significant decrease in the number of effused joint count. As a results, these patients were classified as having mild inflammation with no active clinical disease and were considered excellent responders to the treatment.

## Figure 8.1: Improvement of ESR value in IA patients receiving infliximab

Eight patients with inflammatory arthritis were measured for ESR before and after the therapy. In healthy individuals, the ESR value is 0-15 mm/h in male and 0-20 mm/h in female. ESR is an indirect indicator of inflammation, and is often used as a measure of treatment efficacy in RA patients


125
100
75
50
25
0
ESR (mm/h)
Pre therapy
Post therapy
43
44
45
46
47
48
49
50
Case

### 8.3.2 Effect of infliximab on plasma NO, NOS II, and PKC-η expression in MDM from IA patients

In light of the unexpected results obtained for case 43, we performed similar experiments in seven more patients (cases 44-50) with IA (5 RA and 2 SpA), who were thought to have been receiving the same therapy. As before, we evaluated levels of NO in the plasma samples. As seen in case 43, despite showing improvement in the inflammatory status (*e.g.,* ESR) after the therapy, all seven patients still had elevated serum NO levels (256.2 ± 41.3 μM) (Fig. 8.2), and these were significantly higher than those seen in OA patients (Fig. 7.1) or healthy individuals (143.8 ± 12.5 μM). In certain cases where we had enough MDM generated for the experiments, we evaluated the presence of NOS II expression to see if the elevation of NO in the circulation might be attributed to the monocyte NOS II gene being transcriptionally active. As shown in Figure 8.3 A, cases 46 and 49, but not 48, were positive for NOS II similar to what we had observed previously with case 43. In subsequent screening of PKC-η in MDM from these patients (Fig. 8.3 B), we found that all three cases 46, 48 and 49 were positive for PKC-η. This means that in a total of five cases that we tested (cases 43, 46, 48, 49 and 50. The data from case 50 are to be documented separately below), MDM from three of them expressed both PKC-η and NOS II, and this was consistent with the elevated plasma NO levels we had observed (Table 8.1). Incidently, the level of expression

**Figure 8.2: Plasma NO levels in IA patients receiving infliximab**

NO was measured by assessments of nitrite and nitrate from 50 µL of fresh plasma from healthy or patient volunteers on infliximab therapy. Plasma nitrate was first reduced by nitrate reductase to nitrite, which was then measured by the Griess assay. Each dot represents a case and the mean ± SD is indicated. Statistical analyses were done using unpaired Student's *t*-tests ($p = 0.016$).

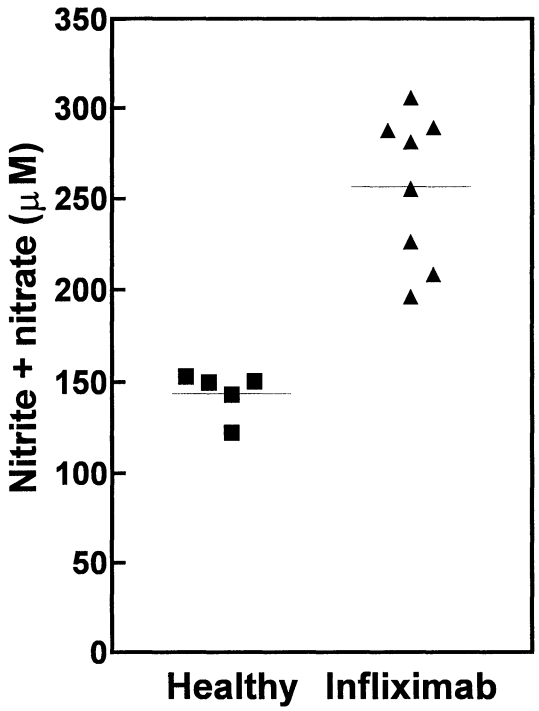
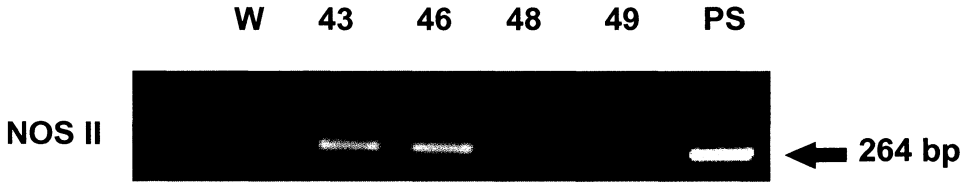

350
300
250
200
150
100
50
0
Nitrite + nitrate (μM)
Healthy
Infliximab

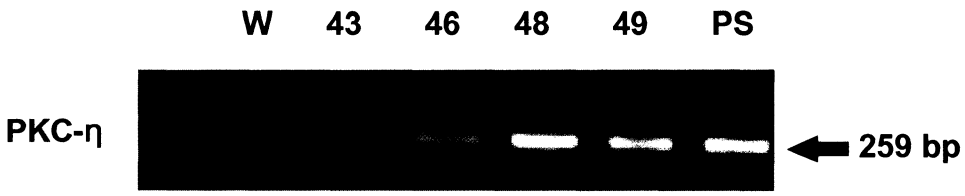
**Figure 8.3: Expression of NOS II and PKC-η in MDM from patients receiving infliximab**

RNA was extracted from MDM , and 2 µg of total RNA was used in amplification by RT-PCR with gene-specific primers. A sample of water (W) in place of test cDNA was used as a negative control, while that of a recombinant NOS II or PKC-η gene fragments served as positive control (PS). PCR products (9 µL) were electrophoresed on a 1% EtBr-containing agarose gel, and the bands visualized were digitally photographed. The positive signals showed bands of the expected sizes for (**A**), NOS II (264 bp) and (**B**), PKC-η (259 bp).

A




B



was shown to be comparable between the patients. Interestingly, an anomaly was also observed in this group. Despite showing moderately high NO levels in the plasma (226.2 μM), MDM from case 50 was completely negative for both NOS II and PKC-η gene. Subsequently, it was revealed that this individual was not receiving infliximab but anakinra instead. As discussed previously, anakinra targets IL-1 function. This finding lead us to examining seven more patients receiving this same therapy to ascertain if the data reflects a change in cell function as a result of IL-1 blocking.

### 8.3.3 Disease activity following therapy with anakinra

As was the case with the infliximab-treated patients, the individuals receiving anakinra also demonstrated improvement in both swollen joint count and the ESR value (Fig. 8.4). Prior to the therapy, all had significantly high ESR value ranging from 30 to 85. However, in response to the treatment all showed a dramatic drop in the ESR. Except in cases 54, 56, and 57, the post-therapy value was comparable to that in healthy adults. In addition, clinical assessments for evidence of joint inflammation at the time sample collection allowed for the determination by the attending rheumatologist that the patients were doing well clinically relative to how they were before the therapy.

**Figure 8.4: Improvement of ESR values in IA patients receiving anakinra**

Eight patients with inflammatory arthritis were measured for ESR before and after the therapy with anakinra. The normal ESR value is 0-15 mm/h for adult male and 0-20 mm/h for adult female.

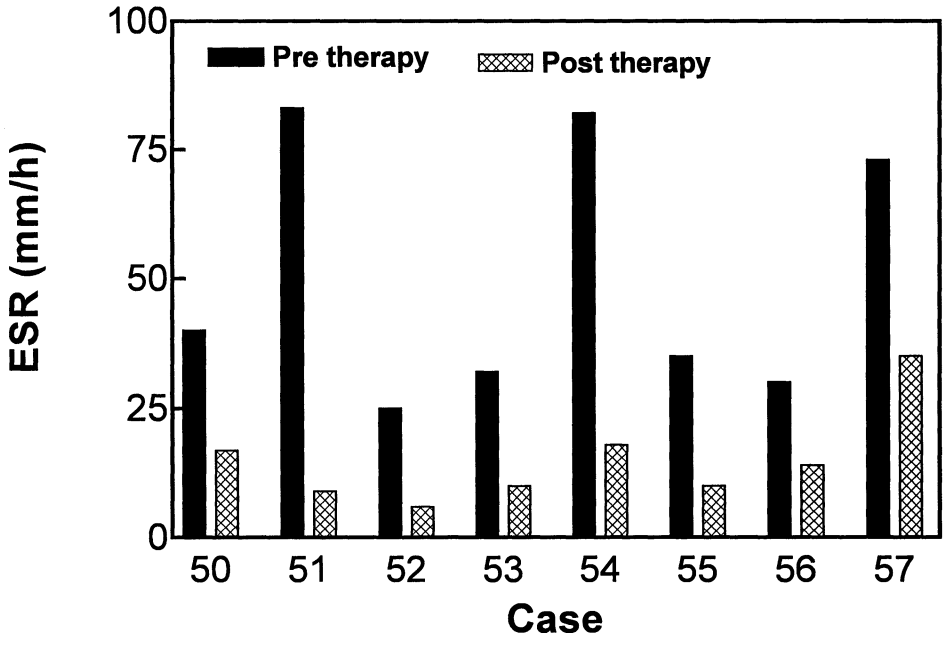

100
75
50
25
0
ESR (mm/h)
Pre therapy
Post therapy
50
51
52
53
54
55
56
57
Case

**Figure 8.5: Plasma NO levels in arthritis patients receiving anakinra**

NO was measured by indirect assessments of nitrite and nitrate from 50 µL of fresh plasma from healthy or patients receiving anakinra. Plasma nitrate was first reduced by nitrate reductase to nitrite, which was then measured by the Griess assay, as described. Each dot represents a case and the mean ± SD is indicated. Statistical analyses were done using unpaired Student's *t*-tests ($p = 0.62$).

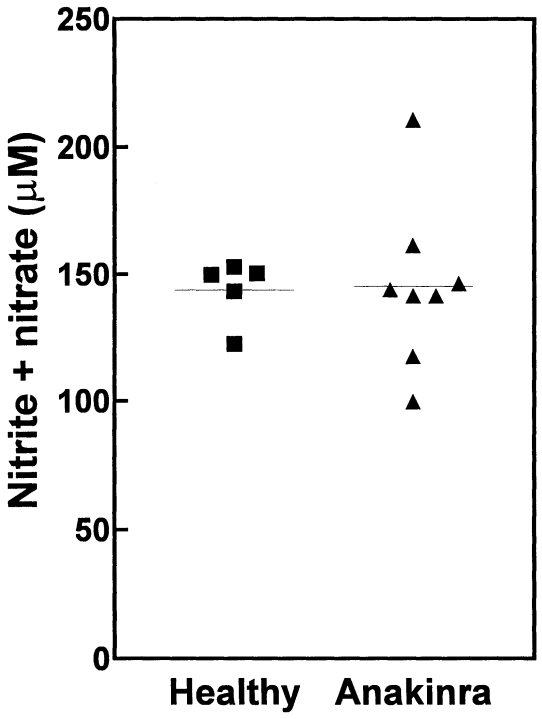

250
200
150
100
50
0
Nitrite + nitrate (μM)
Healthy
Anakinra

**Figure 8.6: Effect of anakinra on NOS II and PKC-η expression in MDM from patients with inflammatory arthritis**

MDM from healthy individuals and arthritic patients were isolated, cultured and assessed for (**A**), NOS II and (**B**), PKC-η expression by RT-PCR using gene-specific primers. A mock sample (W) (water in place of test cDNA) was used for contamination control and a sample of recombinant NOS II or PKC-η fragment served as positive controls (PS). PCR products (9 μL) were electrophoresed on a 1% EtBr-containing agarose gel, and the bands visualized digitally photographed. The positive signals showed the expected bands of 264 bp (for NOS II) and 259 bp (for PKC-η).


A
W 50 51 52 53 54 55 56 57 PS
NOS II
264 bp
B
W 50 51 52 53 54 55 56 57 PS
PKC-η
259 bp

**Table 8.1: Evaluation of plasma NO and co-expression of NOS II and PCK-η in MDM from arthritis patients receiving infliximab and anakinra**

| Subject (ID#) | Age | Sex | $D_x$ | Activity | Steroids | DMARD | NO (μM) | PKC-η | NOS II |
|---|---|---|---|---|---|---|---|---|---|
| **Infliximab-treated** | | | | | | | | | |
| 43(016) | 72 | F | RA | Mild | N | N | 289.2 | Y | Y |
| 44(039) | 41 | M | SpA (AS) | Clinically quiet | N | N | 208.4 | N/D | N/D |
| 45(040) | 72 | F | RA | Mild | N | MTX | 305.7 | N/D | N/D |
| 46(042) | 39 | F | RA | Mild | Y | MTX | 281.2 | Y | Y |
| 47(043) | 43 | F | SpA (PsA) | Mild | N | MTX | 196.1 | N/D | N/D |
| 48(044) | 58 | F | RA | Mild | Prednisone | MTX | 255.2 | Y | N |
| 49(045) | 78 | F | RA | Mild | N | MTX | 287.8 | Y | Y |
| **Anakinra-treated** | | | | | | | | | |
| 50(041) | 36 | F | RA | Mild | N | MTX | 226.2 | N | N |
| 51(046) | 36 | M | RA | Mild | Prednisone | MTX | 141.4 | Y | N |
| 52(047) | 64 | F | SpA (AS) | Clinically quiet | Prednisone | MTX | 141.4 | Y | N |
| 53(048) | 57 | F | RA | Mild | Prednisone | MTX | 143.8 | Y | N |
| 54(049) | 36 | F | RA | Mild | Prednisone | MTX | 161.5 | Y | N |
| 55(050) | 39 | F | RA | Mild | Prednisone | MTX | 210.5 | Y | N |
| 56(051) | 43 | F | SpA (PsA) | Mild | N | MTX | 146.2 | Y | N |
| 57(052) | 42 | F | RA | Mild | Prednisone | N | 117.3 | Y | N |

N/D, not done

### 8.3.4 Effect of anakinra on plasma NO, NOS II and PKC-η expression in MDM from IA patients

As in the case of patients treated with infliximab, we evaluated the levels of circulating NO and studied expression of NOS II and PKC-η in MDM from these IA patients. As shown in Figure 8.5, NO levels in the circulation of these patients (145.2 ± 32.5 μM) were within the same range as healthy people (143.8 ± 32.5 μM), and significantly lower than those seen in infliximab receiving individuals (256.2 ± 41.3 μM). When MDM from these cases were screened for the presence of NOS II, we found no evidence to support the expression (Fig. 8.6 A). Validity of the experiment was ensured by the inclusion of a positive control, a recombinant NOS II fragment; and a negative control, a mock sample in which test cDNA was replaced by water. Surprisingly, unlike what had been observed in case 50, MDM from all of these seven patients were tested positive for PKC-η (Fig. 8.6 B). The data reported in this chapter are summarized in Table 8.1.

## 8.4 SUMMARY AND DISCUSSIONS

Our data presented here show the different effects of infliximab and anakinra on circulating NO and NOS II expression in MDM from IA patients. All four tested patients, who were treated with the TNF-α blocker, showed persistent elevated levels of NO and expressed PKC-η in MDM. Three of the four individuals were also positive for NOS II mRNA in these cells. In contrast, NO levels in those patients,

who were treated with the IL-1Ra, were comparable to those seen in healthy controls or in patients with OA. In accordance with the NO data, there was no evidence for NOS II gene transcription in MDM from any of these individuals. Surprisingly, seven of the eight patients tested were reactive for PKC-η.

The observation in the infliximab-treated group was unexpected since the individuals had shown good response to the therapy and were doing well clinically (as evident from the low score of inflamed joints as well as a substantial decrease in the ESR value). Despite the surprising observation in regard to the NO levels in the infliximab-treated patients, this investigation of the two groups of patients patients has supported our hypothesis that PKC-η appeared to be required for the development of human monocytic cells into a phenotype capable of expressing NOS II upon activation with endotoxin. Simply put, one can infer that when NOS II is expressed in monocytic cells, PKC-η is also present in the same cell type. At this point, we don't have any concrete explanation for the expression of PKC-η in human MDM, and further experimentation needs to be carried out before one can appreciate its significance. We can only speculate that some signals, which are activated during the course of the anakinra therapy, may influence directly, or otherwise, the expression of PKC-η in these patients. As for the two cases where the co-expression of PKC-η and NOS II was not found, it is impossible to draw any useful conclusions from the data, as the sample number was so small. However, it is feasible to offer some scenarios. First, the elevation of NO could be attributed

to other sources, such as the vascular endothelium or inflammatory joints themselves. Second, it is also possible that the genetic makeup of these two patients is such that NOS II is degraded more quickly than the turn-over of NO. Although the pathways leading to the induction of TNF-α and IL-1β in monocytic cells are related, they are distinct in that some essential transcription factors are unique and not shared by the two pathways. It is conceivable that IL-1β and TNF-α might have different effects on how PKC-η is regulated in monocytic cells.

It has been documented that expression of NOS II in macrophages is upregulated in patients with inflammatory conditions like RA suggesting that potential overproduction of NO as a result of the upregulation may contribute to tissue destruction manifested in advanced stages of the disease (Abramson *et al.*, 2001). Therefore, suppression of NOS II expression would offer therapeutic opportunity to the treatment of RA. The work by Perkins *et al.* (1998) suggested that RA patients receiving cA2, which is also a monoclonal antibody to TNF-α, showed a decrease in NOS II enzymatic activity in PBMC, as measured by the levels of L-citrulline accumulated. Therefore, the authors hypothesized that TNF-α would also be involved in the regulation of the NOS II gene. However, our data presented here appear to suggest that suppression of TNF-α with infliximab may not play a role in pre-transcriptional regulation of NOS II, as shown by persistent accumulation of NOS II mRNA and NO in sera of inflammatory arthritis patients receiving infliximab. At the first glance, it seems as if the results from the two

studies contradict each other but there are several possible explanations for the discrepancy. First, the difference could be due to the fact that although patients in the two studies were receiving the same therapy, the drugs themselves were different (infliximab and cA2). It is, therefore, possible that they may have different effects on different patients. Second, while Weinberg's group (Perkins *et al.*, 1998) used PBMC in their study we opted for monocytic cells. We chose to study peripheral blood monocytes for two reasons. First, monocytic cells are the principal cells in the mononuclear fraction containing the NOS II antigen. Second, our *in vitro* data from a previous study suggested that PKC-η may be required for the induction of NOS II and production of NO by LPS in human monocytic cells. We, therefore, wished to test this hypothesis further in MDM from inflammatory arthritic patients in an aim to establish an association between NOS II and PKC-η co-expression *in vivo*. Third, there has been increasing evidence that NOS II, especially in disease processes, can also be produced by B and T lymphocytes (Roman *et al.*, 2002; Schweyer *et al.*, 2000). The fact that Perkins and colleagues used PBMC instead of monocytes raises a strong possibility that the source of NOS II they measured may not be from monocytic cells entirely. In this case, it is possible that NOS II expression may be regulated differently in different cell types, and with that we speculate that a true effect of TNF-α on the regulation of NOS II in monocytes may be masked by that of TNF-α on lymphocytes.

It has been previously proposed that in rheumatoid arthritis, IL-1 primarily

exerts its function locally, within the joint, while TNF-α has a more systemic effect (Dayer, 2002). However, from the perspective of NO release and NOS II expression, our data reported seem to support the reverse. Obviously, further experimentation needs to be performed before the phenomenon can be understood. One approach would be to test sequential samples (from both plasma and MDM) for NOS II in MDM and circulating NO from arthritis patients before and after anakinra therapy. If NO goes back to the normal level and this is accompanied by simultaneous disappearance of NOS II in MDM, the hypothesis of IL-1 being involved in the regulation of NOS II and it having a systemic effect in RA would be greatly strengthened.

# CHAPTER NINE

# PERSPECTIVES AND FUTURE DIRECTIONS

## 9.1 PERSPECTIVES

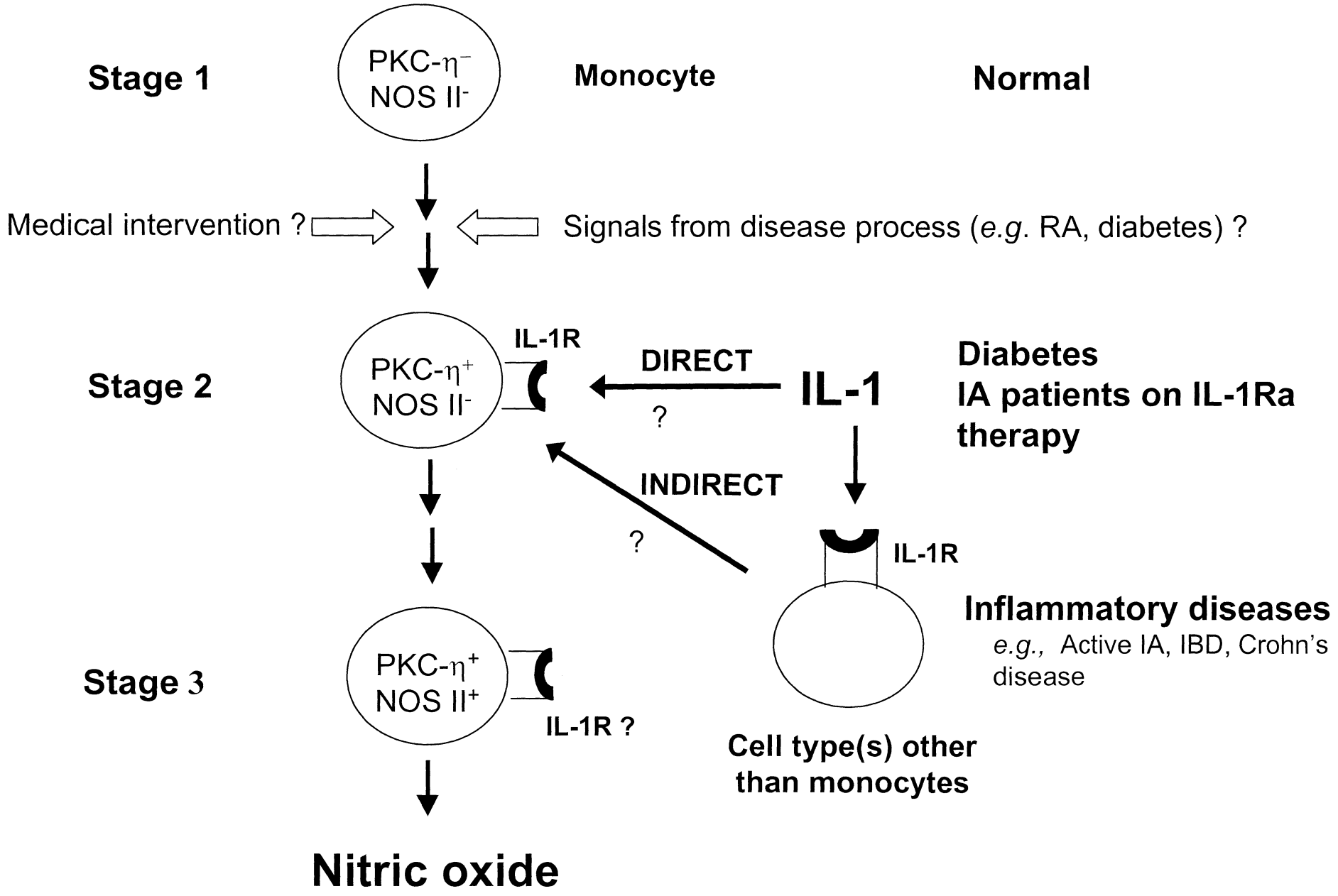
In this project, we investigated the comparative expression of the entire PKC family in both human and murine monocytic cells, and found complete absence of PKC-η in the human cells (Fig. 5.1 and Table 5.1). Subsequent transfection of this gene into human monocytic MM6 cells resulted in NOS II expression by these cells, following their activation with LPS (Fig. 5.6). This finding was not seen in untransfected cells or those transfected with the empty vector. Therefore, the data suggest that in an LPS-triggered signalling pathway, PKC-η is in some way involved in the regulation of NOS II expression in human monocytic cells. This finding may offer important insights into the long standing observation that in normal conditions human monocytic cells can not be induced by LPS readily *in vitro* to express NOS II and release NO. The results obtained from the transfection experiments prompted us to ask if the findings would bear any clinical relevance. To answer this question, we carried out similar investigations in plasma and MDM samples from IA patients. We, subsequently, found that NO levels in plasma from patients with severe disease were significantly elevated (Fig. 7.2). Also, in the same patients whose plasma NO was among the highest, there was evidence for the presence of NOS II mRNA in MDM (Fig. 7.3) suggesting that the transcripts were active. This status of the NOS II transcripts indicates that the source of NO elevation was likely to be from NOS II expressed in MDM. In addition to the presence of NOS II mRNA, we also found transcripts for PKC-η in MDM from the

same patients (Fig. 7.4). It is important to point out that in majority of the cases, when MDM were negative for PKC-η, they were also negative for NOS II mRNA. Indeed, such correlation in expression between NOS II and PKC-η was observed in more than 90% of cases tested. In short, the data from this clinical study confirmed our earlier *in vitro* data and strengthened our hypothesis that PKC-η may be required before human monocytic cells can express NOS II (following their activation with LPS).

Taken together, we propose the following sequential phenotypic changes of human monocytic cells from being double negative for PKC-η and NOS II to being double positive for the two genes. This process is likely to occur in severely affected IA patients (Fig. 9.1). First, we have shown (Fig. 5.1 and Table 5.1) that circulating monocytes from healthy individuals do not express PKC-η (stage 1). Second, signals (not yet identified), which are produced either spontaneously during certain disease processes or as a result of medical intervention, may stimulate transcription of PKC-η in the monocytic cells (stage 2). This is supported by our findings that PKC-η was present in MDM isolated from the three diabetic patients (data not shown) as well as from seven out of eight IA patients, who received the IL-1Ra anakinra (Fig. 8.5). Upon activation with appropriate stimuli, these PKC-η positive monocytic cells express NOS II (stage 3) and, subsequently, release NO locally or into the circulation.

The normalization of NO levels observed in the plasma of the patients

**Figure 9.1: Possible mechanism for the development of PKC-η positive phenotype in human monocytes of IA patients with active disease**



receiving anakinra allows us to propose the following scenario for a role of IL-1 in the aforementioned phenotypic changes of human monocytic cells. On one hand, IL-1 may have an direct role in the transition from stage 2 to stage 3. This would involve IL-1 binding to the receptor, which is present only on PKC-η-positive cells, and triggers a signalling cascade leading to NOS II gene expression. In fact, the notion of IL-1 promoting NOS II expression (see below) was recently illustrated in a study with cartilage isolated from OA and RA patients; attenuation of NO production was found in cartilage cultures previously treated with an IL-1Ra (Vuolteenaho *et al.*, 2003). In addition, it is also feasible to vision an indirect involvement of IL-1 in the phenotypic change of human monocytic cells from stage 2 to stage 3. Binding of IL-1 to its receptor on cells other than monocytes/ macrophages may induce a release of factors which, by binding to their appropriate ligands on PKC-η-positive monocytic cells, can initiate progression to stage 3 from stage 2.

Extracts from the TwHf plant, which is found naturally in China, have been used as traditional medication for thousands of years for a number of autoimmune diseases including inflammatory arthritis. Over the years, substantial effort has been devoted to the purification and characterization of the individual components of the extracts. In this project, two TwHf synthetic derivatives (JPK-101 and JPK-109) and one natural compound (JPK-113) isolated from the root extract were investigated for their effects on the release of inflammatory mediators NO and

TNF-α, as well as NOS II synthesis by monocytic cells. Such investigation was important because elevated levels of these molecules are often associated with the inflammatory response seen in a number of diseases (Abramson *et al.*, 2001; Bingham, 2002; Brantdt *et al.*, 2000). The data have now provided us with several important insights on how these TwHf compounds function. First, the drugs, by themselves have no effect on the cells in terms of mediating default NOS II expression and NO production. Rather, they lead to down-regulation, albeit at varying extent, of the induction of NOS II enzyme as well as that of TNF-α and other cytokines, thereby further strengthening the observed anti-inflammatory characteristic of these agents. More importantly, the fact that the suppressive effects were mainly seen at doses well below the toxic concentration indicates that the findings were not an artefact and could not be attributed to a bystander effect of cell death. Third, although the effects of JPK-109 and JPK-113 on the induction of MMP by LPS in monocytic cells were not investigated in these studies, JPK-101 was not found to have any inhibitory effect on MMP-2 and -9 activity in human LPS-activated monocytic cells. This suggests that the LPS-triggered signalling pathway leading to induction of these two MMP and those giving rise to NO and TNF-α production are distinct from one another.

## 9.2 FUTURE DIRECTIONS

### 9.2.1 Mechanism of action for PKC-η and its involvement in the signalling pathway leading to NOS II expression

Although findings from both experimental and clinical studies presented in this thesis have revealed an apparent involvement of PKC-η in the regulation of NOS II expression in monocytic cells, it would be prudent to carry out further experimentation to delineate exactly where it is along the NOS II pathway that PKC-η most likely to carry out its function. Determination of its substrate(s) by phosphorylation studies would also shed light on what molecules might be possible targets for the kinase.

### 9.2.2 Possible connection between PKC-η and IL-1: a role for IL-1 in the regulation of NOS II expression in monocytic cells

The expression of NOS II and PKC-η in MDM from patients receiving the monoclonal anti-TNF-α antibody, infliximab, was markedly different from that in those receiving anakinra, which targets IL-1 function. With the continuing advancement of technology, it would be feasible to perform gene expression profile using microarray analysis to ascertain which set of genes might be expressed in each case. This might offer important clues as to what might be involved in turning on the expression of PKC-η in the anakinra receiving group. With respect to the proposed role of IL-1 in the development of stage 3 phenotype, the following

studies could be carried out in severely affected IA patients to test the hypothesis. First, expression of the IL-1 receptor (IL-1R) on the MDM cell surface from PKC-η expressing cells can be assessed by flow cytometry. If, the presence of IL-1R is only found on PKC-η expressing cells, this would suggest a direct involvement of IL-1 in cell differentiation from stage 2 to stage 3 in that the binding of IL-1 to IL-1R mediates initiation of a signalling cascade leading to expression of NOS II enzyme. The other possibility from the IL-1R screening experiment would be the absence of IL-1R on monocytic cells which express PKC-η. In this instance, the data generated from the current work will likely suggest that the proposed IL-1 involvement is indirect. That is, binding of IL-1 to its receptor on another cell type may induce production of other factor(s), which themselves bind to their receptors present on the PKC-η expressing cells, and initiate a necessary cascade leading to NOS II expression.

In regard to a possible relationship between IL-1 and NOS II, the findings from our study with the anakinra receiving patients offer a possibility that IL-1 is a positive regulator of NOS II expression in human monocytic cells. Indeed, previous findings have shown that NOS II enzyme could be induced in human chondrocytes following IL-1 stimulation (Charles *et al.*, 1993) and that *in vitro* NO production by cartilage explants from arthritic patients was attenuated in the presence of IL-1 receptor antagonist (Vuolteenaho *et al.*, 2003). Whether these observations also hold true in monocytic cells remains to be determined. Thus, evaluation of

sequential samples (serum and MDM) from IA patients before and after therapy might help support or refute this hypothesis. Specifically, if co-expression of NOS II and PKC-η in MDM (and elevated NO levels) seen in severely affected patients before the therapy is no longer the case in the same patients after the therapy, this would support a positive involvement of IL-1 in the LPS-mediated pathway leading to NOS II induction.

Our findings with three diabetics (data not shown) and with the anakinra-treated IA patients (Chapter 8) show the presence of PKC-η gene transcription in MDM. This offer an *in vivo* model for further assessment of correlation in expression between PKC-η and NOS II. If exposure of these PKC-η-positive cells to LPS leads to induction of NOS II expression and subsequently, to NO production, this would add significant strength to the proposed hypothesis that PKC-η protein has to be present before LPS activated human monocytic cells could express NOS II.

### 9.2.3 Future work with TwHf compounds

It would be useful to conduct a time course study to ascertain the time point(s) at which these two compounds are most active. This piece of information would certainly provide important insights into the target molecules that are involved in the NOS II and TNF-α signalling pathways. Now that we have established that the three TwHf compounds significantly attenuate NOS II

expression in LPS-activated monocytes, and that PKC-η appears to be required for the development of NOS II-positive phenotype, it would be very useful to investigate a possibility of the TwHf compounds to inhibit/attenuate NOS II expression and NO production in LPS-activated human monocytes. A positive finding from such a study would greatly strengthen the notion that PKC-η was involved in the regulation of NOS II expression in human monocytic cells.

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