Understanding the molecular mechanism(s) involved in anti-inflammatory and anti-tumor effects mediated by α-Melanocyte Stimulating Hormone and Mangiferin

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Dedicated to…

my parents

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Declaration

The research work embodied in this thesis entitled, "**Understanding the molecular mechanism(s) involved in anti-inflammatory and anti-tumor effects mediated by α-Melanocyte Stimulating Hormone and Mangiferin**", has been carried out by me at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, under the guidance of Dr. Sunil Kumar Manna. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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Certificate

This is to certify that this thesis entitled, "**Understanding the molecular mechanism(s) involved in anti-inflammatory and anti- tumor effects mediated by α-Melanocyte Stimulating Hormone and Mangiferin**", submitted by Ms. Abira Sarkar, for the degree of Doctor of Philosophy to the University of Hyderabad is based on the work carried out by her at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad. This work is original and has not been submitted for any diploma or degree of any other university or institution.

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Contents

Chapter Six

Mangiferin, a β-D Glucoside suppresses TNF induced cell signaling

ABBREVIATIONS

Abbreviations for standard (SI) units of measurement, and chemical formulae are not included in the list below:

AP-1	Activator protein-1
ATZ	3-amino-1, 2, 4-triazole
BSA	Bovine serum albumin
BSO	L-buthionine sulfoximine
CD95	Fas receptor
CE	Cytoplasmic extract
Ceramide	N-acetyl-D-sphingosine
Cox2	Cyclooxygenase 2
CXCR	Chemokine receptor
DOC	Deoxycholate
Dox	Doxorubicin
DPH	Diphenyl hexatriene
DSS	Disuccinimidyl suberate
DTNB	5, 5-dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EMSA	Electrophoretic mobility shift assay
FasL	Fas ligand
FBS	Fetal bovine serum
FMLP	Formyl methionyl leucyl phenylalanine
γ-GCS	Gamma glutamyl cysteine synthetase
GSH	Glutathione
GSSG	Oxidized form of glutathione
ICAM	Intracellular adhesion molecule
IκBα	Inhibitory subunit of kappa B
IκBα-DN	IκBα dominant negative
iNOS	Inducible nitric oxide synthase
IL	Interleukin

KSCN	Potassium thiocyanate
MC-R	Melanocortin receptor
MDC	Monodansyl cadaverine
MGSA	Melanocyte growth stimulatory activity
Mn-SOD	Manganese superoxide dismutase
α-MSH	Melanocyte stimulating hormone
MTT	3-(4,5-Dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NE	Nuclear extract
NF-κB	Nuclear transcription factor kappa B
NIK	NF-κB-inducing kinase
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PIS	Pre-immune serum
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophils
PMSF	Phenyl methyl sulphonyl fluoride
ROI	Reactive oxygen intermediate
SA-LPS	Serum activated lipopolysaccharide
SEAP	Secretory alkaline phosphatases
TBA	Thiobarbituric acid
TNF	Tumor necrosis factor
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor

Preface.

Inflammation is a physiological response of the body to protect tissues from infection, injury or disease. The inflammatory response begins with the production and release of chemical agents by cells in the infected, injured or diseased tissue. Inflamed tissues generate additional signals that recruit leukocytes to the site of inflammation. Leukocytes destroy any infective or injurious agent, and remove cellular debris from damaged tissue. This inflammatory response usually promotes healing but, if uncontrolled, may become harmful. The statistics of patients suffering from inflammatory diseases is alarming. Almost 50% of individuals above 60 years of age suffer from various forms of arthritis. By 2009, the total number of rheumatoid arthritis sufferers will increase to an estimated 7.2 million. Coronary heart disease (CHD), account for nearly 50% of the deaths in the major pharmaceutical nations of the world. Sepsis ranks as the 10th leading cause of death in the US and kills approximately 1,400 people worldwide every day.

Designing effective drugs to treat such diseases is therefore, one of the major challenges pharmaceutical companies are facing. Even with several effective drugs in the market, the two main problems encountered are excessive side effects and also resistance over time of usage. A strategy that is fast developing in such a scenario is to utilize the body's endogenous mechanism of resolution. Alternatively, drugs based on plant products are also gaining importance. Nevertheless, to successfully handle such diseases one needs to thoroughly understand the body's immune response, the cells and mediators involved. There is also necessity to understand the signaling pathway undertaken by the drugs before they are marketed, to make best use of them. Hence, the aim of the present study was to understand the molecular mechanism (s) involved in the signaling of two such molecules - one an endogenous resoluter called α-Melanocyte stimulating hormone and the other a plant derived polyphenolic compound called Mangiferin, in relation to their anti-inflammatory and anti- cancer activities.

Chapter1 reviews the current understanding of Inflammation, describing the various cells involved in inflammatory responses, some of the potent mediators of

inflammation and the various diseases in which they are implicated. The importance of certain transcription factors and intermediate signaling molecules playing crucial role in inflammation and cancer development and progression are also documented. An overview of importance of endogenous resoluters and significance of plant products in treatment of inflammatory disorders is also presented. Chapter 2 gives a detailed account of the materials and experimental procedures adopted for the various investigations carried out as documented in various chapters.

Receptors of α-MSH were detected on various cells involved in the immune response, hence we aimed to understand the role of α-MSH in modulating the responses in these cells. Chapter 3 mainly deals with how α-MSH downregulates endotoxin (LPS) mediated responses in mast cells. α-MSH was found to inhibit LPS stimulated NF-κB and NF-κB dependent gene expression. Sustained treatment with α-MSH resulted in mast cell death as depicted by increase in ROI generated, lipid peroxidation, caspase8 activation and PARP cleavage. Transcription factor, NF-kB gives a proliferative signal to the cell, hence its inhibition for a sustained period of time might lead to cell death which appears to be the action of α-MSH on mast cells. This work is published as *Sarkar A et al.,* (2003), FEBS Letters. 459: 87-93.

Chapter 4 describes the experiment and results obtained with α-MSH on THP-1 derived macrophage cell line. LPS plays a significant role in sepsis and exerts its action in macrophages mainly via the CD14 receptor. We observed that a-MSH inhibits LPS mediated responses in macrophages like proteolytic enzyme release, NF-kB and its responsive gene activation. These responses were found to be exhibited by downregulation of the CD14 from the surface of macrophages. This part of the work is published as *Sarkar A et al.,* (2003), FEBS Letters. 553: 286-294.

IL-8 is a potent neutrophil chemotactic factor belonging to the CXC family of cytokines and functions by interactions with IL-8 receptors (CXCR1 and CXCR2), which are G-protein coupled receptors. IL-8 elicits multiple effects in inflammation, tumor growth, angiogenesis and metastasis. Chapter 5 aims to reveal

the mechanism of action of α-MSH in modulating IL-8 mediated responses in Neutrophils and macrophages. α-MSH downregulates IL-8 mediated leukocyte migration, oxidative burst response, ROI generation, NF-kB activation and NF-kB dependent gene expression. α-MSH in a mechanism not still understood, led to release of neutrophil elastase leading to the downregulation of receptors of IL-8, CXCR1 and CXCR2 from the Neutrophils cell surface, thereby inhibiting IL-8 mediated responses. This work is accepted for publication and is ahead of print as *Sarkar A et al.,* (2006), Eur. J. Immunol. 36: 754-769.

With the increasing importance of plant based products in treatment of several diseases, we also looked into the signaling of one such polyphenol called mangiferin, which is reported for its remarkable anti-inflammatory, anti-oxidant and anti-tumor activities. TNF-α is a multifaceted cytokine, which is involved in several inflammatory diseases. Chapter 6 describes the action of mangiferin on TNF elicited responses. Mangiferin was found to inhibit TNF activated NF-κB and its gene products, increases the expression of endogenous anti-oxidant Glutathione and the activity of catalase and thus inhibit ROI generated. TNF cuts the life lines of cancer cells but also activates NF-κB, which drives expression of anti-apoptotic genes. Hence, inhibition of NF-κB can potentiated the cell death induced by TNF. Mangiferin also proved successful in potentiating cell death mediated by various other standard chemotherapeutic agents. The work described is published as *Sarkar A et al.,* (2004), J. Biol. Chem. 279: 33768-33781.

Hence, the study presented in this thesis, for the first time reports the different signaling pathways of α-MSH in various cells of the body involved in immune responses and also the mechanism of action of mangiferin in counteracting TNF mediated signaling.

CHAPTER ONE

INTRODUCTION

AND

REVIEW OF LITERATURE

1. Overview of inflammation

Human beings or animals are exposed to a multitude of different pathogens including bacteria, fungi, protozoan or metazoan parasites, viruses, and even an array of harmful chemicals agents, which are all capable of deranging its homeostasis. As a response to combat the wide range of such antigens, the immune system has developed a complex mechanism broadly termed as ***Inflammation***. Inflammation has thus been one of the earliest addressed problems of biology. Study on inflammation dates back to the age between 30BC to 38AD when Celsus described inflammation with its cardinal signs of heat, redness, swelling and pain. Among the few scientists who have made major contributions are Lewis (1881-1945), who described the triple response; Cohnheim and Samuel (Late 1800s), who reported on leukocyte emigration and vascular permeability; and Metchnikoff (1845-1916), who described phagocytosis. Inflammation can be rapid and last a short period of time (acute inflammation), or can persist for a long time due to a continuous stimulus or injury (chronic inflammation). Years of research have led to our understanding of several factors involved in this process. The initial events of inflammation are the results of vasodilation and increased vascular permeability, leading to exudation of fluid and plasma proteins and recruitment of cells of body's defense system including mast cells, platelets and leukocytes. The statistics of patients suffering from inflammatory disorders is alarming. Allergies are the 6th leading cause of chronic disease in the United States. The worldwide prevalence rates of Asthma are increasing, on average, by 50% per decade. One in 10 individuals over 65 and nearly half of those over 85 suffer from various forms of Arthritis. Inflammation is also a cause for cardiovascular diseases and is responsible for nearly 42% of the deaths in the United States alone. Neurodegenerative disorders are estimated to affect over 22 million people worldwide (Alzheimer's Association, 2004). Cancer, which is also now linked to inflammation and leads to more than 7 million deaths worldwide. More than 10 million new cancer cases are diagnosed each year. Cancer claimed twice as many lives as AIDS in 2004. Nevertheless, Inflammation is important as it is the physiological response generated within the body to

respond to the wide range of foreign substances by which the body tries to clear the damage-causing agent and restore the body's homeostasis.

1.1 Outcome of uncontrolled inflammation:

The ability to mount an inflammatory response is essential for survival. However, it is equally essential to have a control over the response generated since excessive or uncontrolled response results in a vast array of diseases (as depicted by the statistical report mentioned above), which include some of the highly prevalent diseases such as

- Allergy – including allergic rhinitis, sinusitis, skin allergies (utricaria/hives, atopic dermatitis), food allergies, drug allergies, insect allergies, and rare disorders such as mastocytosis
- Asthma
- Arthritis, including osteoarthritis, rheumatoid arthritis, and spondyloarthropathies
- Autoimmune conditions systemic lupus erythematosus, dermatomyositis, polymyositis, inflammatory neuropathies like Guillian Barre, Vasculiitis.
- Cardiovascular diseases and Cancer.
- Neurodegenerative diseases like Alzheimer's, Multiple sclerosis, and Parkinson's disease.

To regulate the body's response in order to prevent the excessive damage that can be caused, it is essential to first thoroughly understand the key players involved in this response which include the body's defense cells, the mediators released and finally the mechanism involved in inflammatory response.

1.2 Major players of inflammation

Some of the major cells of the body that are responsible for the release of inflammatory mediators and play important role in the development of inflammation are as follows…

1.2.1 Role of Mast cells

Mast cells play a crucial role in inflammation especially in the development of allergic reactions. They originate from CD34+ bone marrow progenitor cells and thereafter mature under local tissue micro environmental factors. They express on their surface, the receptor (FcRI) that binds with high affinity the Fc portion of IgE antibody. Mast cells are normally distributed throughout connective tissues, especially beneath the epithelial surfaces of the skin, in the respiratory system, in the gastrointestinal and genitor-urinary tracts, adjacent to blood or lymphatic vessels, and near or within peripheral nerves (Kitamura Y, 1989; Galli SJ, 1990; Galli *et al.,* 1984; Schwartz and Austen 1984). This distribution places mast cells near parasites and other pathogens as well as near environmental antigens that come in contact with the skin or mucosal surfaces. The location of mast cells also facilitates their exposure to products of biting or stinging insects, to blood-borne antigens and other substances that pass into the interstitium, and to neuropeptides. There are two category of inflammatory mediators in mast cells- the ***Preformed mediators***, stored in secretory granules and secreted upon cell activation, which include a biogenic amine, typically histamine, proteoglycans, either heparin, over-sulphate chondroitin sulphates or both, and a spectrum of neutral proteases and a range of ***Newly formed mediators*** which include multifunctional pro-inflammatory cytokines, like interleukin-1, 3, 4, 5, and 6; granulocyte-macrophage colony-stimulating factor GM-CSF; interferon gamma IFN-g; four members of the C-C branch of the intercrine family (macrophage inflammatory proteins 1, T-cell-activation antigen 3, and tumor necrosis factor-α (TNF-α) (Plaut *et al.,*1989; Wodnar-Filipowicz *et al.,*1989; Burd *et al.,* 1989). In aggregate, these cytokines and thus the mast cells not only regulate IgE production and other immune responses, but also affect inflammation, homeostasis, hematopoiesis, angiogenesis, tissue remodeling, and tumor development or resistance (Galli *et al.,* 1991).

1.2.2 Role of Monocytes and Macrophages

Monocytes and macrophages were classified cells of the Reticuloendothelial system (RES). Their development takes place in the bone marrow and passes through the

following steps: stem cell- committed stem cell- monoblast- promonoblast-monocyte (bone marrow)-monocyte (peripheral blood)-macrophage (tissues). Blood monocytes possess migratory, chemotactic, pinocytic and phagocytic activities, as well as receptors for IgG Fc domains (FcγR) and C3b complement. They undergo differentiation in the tissues to give rise to multifunctional tissue macrophages. Hence monocytes are considered as immature macrophages. Macrophages are one of the important players in inflammation, which take part in antigen presentation, phagocytosis and immunomodulation through production of various cytokines and growth factors. They play a critical role in the initiation, maintenance and resolution of inflammation. Activation signals include cytokines (IFN-γ), GM-CSF and TNF-α, LPS, extra cellular matrix proteins and various chemical mediators. These cells are involved in several pathologies. Monocytes and macrophages are involved also in normal and physiological adult angiogenesis (Moldovan NI, 2002). Alveolar macrophages play important role as orchestors of inflammation in chronic obstructive pulmonary disease (COPD). Their number is markedly increased in these patients due to increased recruitment, proliferation and survival. Alveolar macrophages show increased secretion of inflammatory mediators, oxidants, proteins and proteinases in patients with COPD and hence novel strategies to target macrophages in these disorders are evolving (Barnes PJ, 2004). Macrophages are reported to be involved in demyelination of neurons, a pathological hallmark of neuropathies like Guillian Barr Syndrome. They are recruited along with T cells to the neurons with the concerted action of adhesion molecules, matrix metalloproteases, chemotactic signals, resulting in the production of pro-inflammatory cytokines and auto-antibodies mediated attack of the myelin sheath all leading to the demyelination process (Keifer *et al.,* 2001).

Tumor associated macrophages are found to be responsible for poor prognosis in many solid tumor types. These are attracted to the sites by over expression of macrophage chemo attractant proteins secreted by the tumors and in turn macrophages secrete cytokines and growth factors needed for the cancer progression and metastasis. Mouse

deficient in macrophages showed reduced progression of tumors and metastasis (Lin and Pollard, 2004). Thus macrophages can become an important target in cancer therapy.

1.2.3 Role of Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes (PMN), represent 50 to 60% of the total circulating leukocytes and constitute the "first line of defense" against infectious agents or ‘‘nonself’’ substances. They are the first cells to be recruited to sites of infection or injury. Their targets include bacteria, fungi, protozoa, viruses, virally infected cells and tumor cells. The major role of neutrophils is to phagocytose and destroy infectious agents but they also limit the growth of some microbes, thereby buying time for adaptive (specific) immunological responses. The Neutrophils-mediated inflammatory response can be regarded as a multi-step process involving the initial adhesion of circulating neutrophils to activate vascular endothelium, subsequent extravasation and migration of neutrophils towards inflammatory foci, and the ultimate in situ elimination of foreign microorganisms through phagocytosis, generation of reactive oxygen metabolites, and release of microbicidal substances. Most of the steps in this process are dependent on the mobilization of cytoplasmic granules and secretory vesicles. The various subsets of granules contained within the neutrophil constitute an important reservoir of anti-microbial proteins, proteases, and components of the respiratory burst oxidase, a wide range of membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins, bacterial products, and soluble mediators of inflammation. Neutrophil granules are formed sequentially during myeloid cell differentiation. Formation of granules (granulopoiesis) is initiated in early promyelocytes, when immature transport vesicles bud off from the Golgi and fuse together (Bainton *et al.*, 1971; Hartmann *et al.*, 1995). Granules formed at a given stage of myelopoiesis are packaged with proteins synthesized simultaneously, and granule heterogeneity arises secondarily to differences in the biosynthetic windows of granule proteins. The granules are therefore named as Azurophil granules, Specific granules, Gelatinase granules and Secretory vesicles based on their contents. For example, *Azurophil granules* mainly contain myeloperoxidase,

alpha-defensins, serprocidins (serine proteases with microbicidal activity): proteinase-3, cathepsin G, and elastase. The serprocidins are cationic polypeptides of 25–29 kDa (Campanelli *et al.,* 1990; Salvesen *et al.,* 1987; Sinha *et al.,* 1987), which display proteolytic activity against a variety of extracellular matrix components, such as elastin, fibronectin, laminin, type IV collagen, and vitronectin. Furthermore, they induce activation of endothelial and epithelial cells, macrophages, lymphocytes, and platelets, and possess antimicrobial properties (Owen *et al.,* 1999). Unrestrained elastase activity is currently believed to play a crucial role in the pathogenesis of pulmonary emphysema, especially in patients with underlying α1-antitrypsin deficiency (Witko-Sarsat *et al.,* 2000). These proteases participate in hydrolysis of extracellular proteins and cell surface receptors (Porteu *et al.,* 1991).

Although neutrophils are essential for host defense, they have also been implicated in the pathology of many chronic inflammatory conditions and ischemia-reperfussion injury. Under normal conditions, blood may contain a mixture of normal, primed, activated and spent neutrophils. In the inflammatory site, mainly activated and spent neutrophils are present. Activated neutrophils have enhanced production of reactive oxygen intermediates (ROI). A subpopulation of neutrophils with the enhanced respiratory burst has been detected in the blood of people with an acute bacterial infection and patients with the adult respiratory distress syndrome (ARDS). The acute phase of thermal injury is also associated with neutrophil activation, and this is followed by a general impairment in various neutrophil functions. Activation of neutrophils by immune complexes in synovial fluid contributes to the pathology of rheumatoid arthritis. Chronic activation of neutrophils might also initiate tumor development because some ROI generated by neutrophils damage DNA and promote tumor cell migration. Further research into granules and their constituents may allow identification of novel antibiotics for clinical use and elucidate the pathogenesis behind neutrophil-mediated inflammatory disorders. Development of drugs that regulate the release of the neutrophil granule contents might also be another strategy to control neutrophil driven inflammatory diseases.

1.3 Important mediators of inflammation

The cells of the immune system mediate their action with the help of several mediators. Some of the important ones recognized in the pathology of various diseases are as follows.

1.3.1 Cytokines like Tumor Necrosis Factor-α (TNF-α)

TNF is a multifaceted cytokine produced in response to infection or immunological injury and mediates several responses, which extend from its well characterized pro-inflammatory properties to diverse signals such as cellular differentiation, proliferation and death (Vassalli P, 1992; Vandenabeele *et al.*, 1995). The wide range of roles played by TNF is attributed to the differential bioactivities of its soluble and transmembrane form (Grell *et al.*, 1995) and the differential functioning of its two tumor necrosis factor receptors (TNFR I and TNFR II). TNF has been established to have a dominant role in pathogenesis of chronic inflammation and autoimmunity.

On one hand TNF has **beneficial effects** with its ability to:

A) Suppress tumor growth from where it derives its name. TNF is found to also increase the uptake of vasoactive drugs like melphalan and doxorubicin adding to its potential as an anticancer agent. However, due to its high systemic toxicity, the use of TNF as an anti-cancer drug is limited.

B) TNF and its family members regulate the immune system by virtue of their ability to induce apoptosis of cells since immune system regulation is not only by cell proliferation and differentiation but also by the effective programmed cell death mechanism (apoptosis) to eliminate out the unwanted cells. C) TNF also has been shown to protect cells from infection against *Listeria monocytogenes* and also plays role in protecting against *Mycobacterium tuberculosis*.

On the other hand the **deleterious effects** of TNF include:

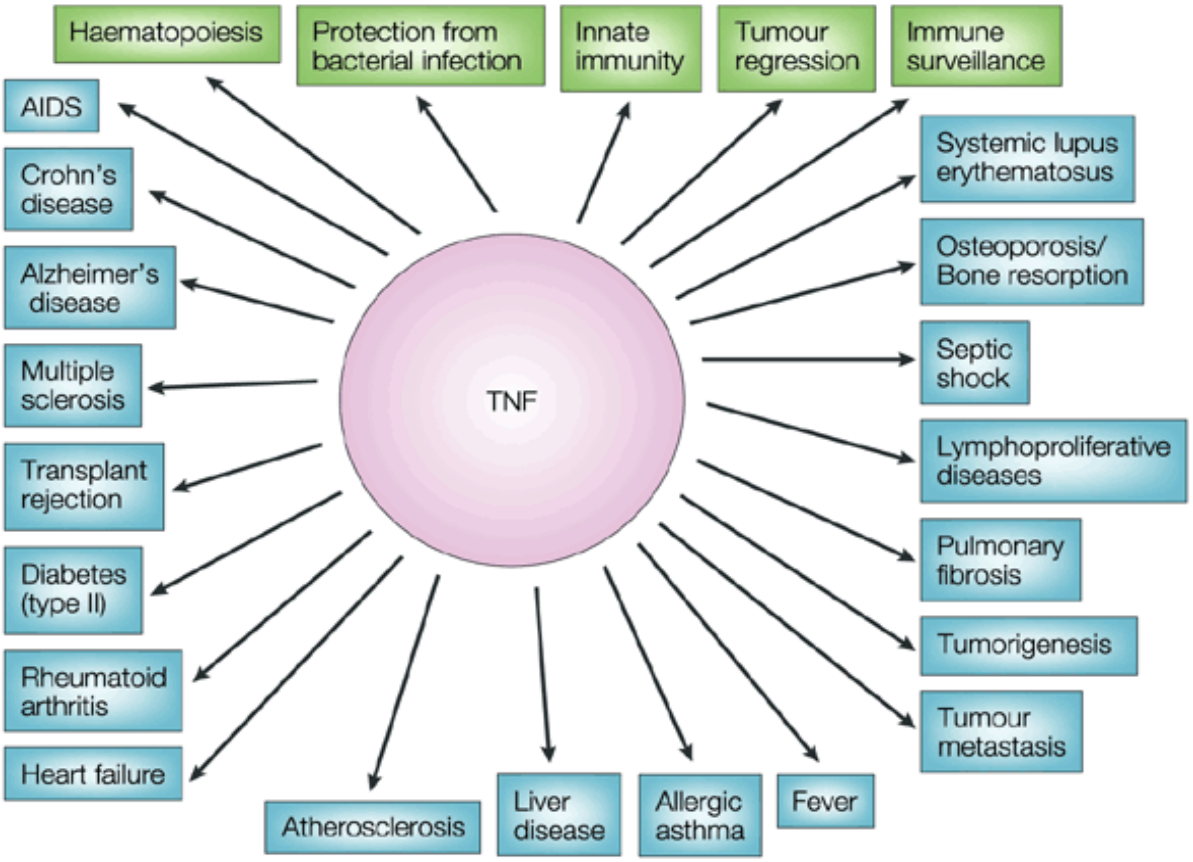
A) *Cancer progression*: TNF is well documented to act via the NF-κB pathway. Activation of NF-κB results in expression of several genes that are involved in invasion and metastasis, including adhesion molecules, matrix metalloproteinases (MMP), COX2

and vascular endothelial growth factor (VEGF). Activation of NF-κB suppresses apoptosis, enhancing tumerogenicity. Thus, mouse deficient in TNF were shown to be resistant to skin carcinogenesis (Moore *et al.,* 1999). Hence, TNF seems to be an autocrine growth factor for many tumors.

B) *Inflammation*: TNF and its family members are implicated in a wide range of inflammatory disorders including the Rheumatoid arthritis, Inflammatory bowel disease, Multiple sclerosis, Chronic heart failure, Bone resorbtion, Alzheimer's disease, Arthrosclerosis, transplant rejection and hepatoxicity.

C) *Autoimmunity*: TNF and some of its family members have been shown to be involved in the autoimmunity. For example the over expression of B cell activating factor, BAFF (McKay *et al.,* 2003) leads to increased splenomegaly, DNA-specific antibodies, also there is growing evidence of TNF being involved in the pathogenesis of type II diabetes, wherein TNF has been shown to interfere with an insulin-signaling mechanism by inhibiting the tyrosine kinase activities of the insulin receptor substrate, and also leads to up regulation of the expression of suppressor of cytokine signaling (SOCS) (Emanuelli *et al.,* 2001).Thus TNF seems to be an ideal target for several therapeutics and indeed drugs based on TNF and its family members are now well accepted, for example, in Europe TNF has been approved for use in treatment of sarcomas and melanomas (Eggermont *et al.,* 2003), antibodies specific for TNF is approved for treatment of Chron's disease and various cancers (Suenaert *et al.,* 2002). Still, there needs to be further studies to improve the efficacy of these drugs in order to minimize the side effects that are documented.

Various diseases associated with aberrant TNF production



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1.3.2 Role of Lipopolysacharide (LPS)

Sepsis is among the top ten causes of death worldwide. More than 1,400 people die from severe sepsis every day. Sepsis is a systemic inflammatory response syndrome to a localized or systemic infection that leads to the overproduction of proinflammatory cytokines, such as TNF-α, IL-1 and the ultimate failure of multiple organ systems. Lipopolysacharide (LPS), an endotoxin found in the outer membrane of Gram-negative bacteria (Doe *et al.,* 1978), is a major trigger of sepsis. Recognition of LPS is crucial for host antimicrobial defense reactions (Ulevitch *et al.,* 1995; Schletter *et al.,* 1995). LPS stimulate mononuclear cells (monocytes and macrophages) and neutrophils to produce immunoregulatory and proinflammatory cytokines such as IL-1, IL-6, TNF-α, TGF-β, and Prostaglandins (Medvedev *et al.,* 2000; Morrison *et al.,* 1978; Raetz *et al.,* 1991; Dentener *et al.,* 1993). The myeloid differentiation antigen CD14, a 55-kDa glycosylphosphatidylinositol-anchored membrane glycoprotein (mCD14), has been shown to play essential roles in the activation of human mononuclear phagocytes by LPS (Wright, S. D, 1990). CD14 is expressed predominantly on the surface of monocytes, macrophages, and neutrophils, (Dentener *et al.,* 1993; Fearns *et al.,* 1995) and it also exists as a soluble plasma protein lacking the glycosylphosphatidylinositol anchor (sCD14) (Ulevitch *et al.,* 1995). Both forms have been shown to play crucial roles in the recognition of LPS and in the initiation of cellular immune responses by LPS.LPS-binding protein (LBP), a 60-kDa serum glycoprotein produced by the liver, has also been shown to enhance LPS-induced cytokine production by monocytic cells (Corradin *et al.*,1992). LBP binds to the lipid A region of LPS to form an LBP-LPS complex, which then interacts with CD14 to induce cytokine production (Meszaros *et al.,* 1994). Identification of cell surface receptors *e.g.* the Toll-like receptors (TLR), which interacts with the LPS-LBP-CD14 complex, has further elucidated the mechanisms of LPS induced signaling pathways (Schuster *et al.,* 2000). Although CD14 and LBP are involved in LPS signaling (CD14-dependent pathways), the existence of additional signaling pathways (CD14-independent) have been reported by other investigators (Netea *et al.,* 1998). Human polymorphonuclear leukocytes are responsible for killing microorganisms and eliminating

cellular debris. These functions are mediated by superoxide (O_2^-) generated by an NADPH-dependent oxidase. The assembly of NADPH oxidase is up-regulated in neutrophils exposed to bacterial LPS (DeLeo *et al.,* 1998) suggesting a role for LPS priming of the respiratory burst in polymorphonuclear leukocytes. There are also reports suggesting the LPS mediated TNF-α secretion to be Nuclear factor kappa B (NF-κB) dependent (Sanlioglu *et al.,* 2001). Despite extensive research, efficacious therapies for sepsis have yet to be developed (Bone RC, 1996). Moreover, clinical trials using either pharmacological agents or monoclonal anti-endotoxin antibodies have not been successful (Ziegler *et al.,* 1991; McCloskey *et al.,* 1994; Bone RC, 1995). Lack of complete understanding the molecular mechanism of the pro-inflammatory signaling pathway and the action of the drug is one reason for failure of such drugs. Thus studying the molecular mechanism of action of these drugs in detail would definitely be useful to avoid the possible side effects the drug could cause.

1.3.3 Role of adhesion molecules in inflammation

The luminal endothelial cell surface is a relatively nonadhesive and nonthrombogenic conduit for the cellular and macromolecular constituents of the blood. The extracellular matrix holds the basal endothelial cell surface in a well-arranged array. In certain diseases, various adhesive interactions between endothelial cells and the constituents of the blood or extracellular matrix are changed. These diseases include inflammation, atherosclerosis, pathologic angiogenesis, and vascular injury. During these disease processes, adhesion molecules are closely involved (Gimbrone *et al.,* 1997). Inflammatory signals induce the expression of proteins on the endothelial cell surface that promote the adhesion and extravasation of activated immune cells from the circulation into the underlying tissues. Key among these molecules are P- and E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cells, and their respective counter receptors, P-selectin glycoprotein ligand-1 (PSGL-1), leukocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), on the leukocytes. In vitro blockade of these molecules inhibits the adhesion of leukocytes

(Dedrick *et al.,* 2003). Reports have shown the involvement of NF-κB pathway in the expression of the 1CAM-1 and VCAM-1 in cells stimulated by the VEGF and hence the blockade of this pathway could suggest a therapeutic strategy to control inflammation regulated by the cell adhesion molecules (Kim *et al.,* 2001). Adhesion blockade in animal models prevents or ameliorates graft rejection and disease severity in autoimmune models (Dedrick *et al.,* 2003). Clinical studies with humanized monoclonal antibodies, which interfere with LFA-1/ICAM-1 or VLA-4/VCAM-1 interactions have shown significant efficacy and good safety profiles in autoimmune disease, including psoriasis, multiple sclerosis and inflammatory bowel disease. Thus, adhesion blockade is emerging as a useful therapeutic strategy in several inflammatory settings. Early phase of atherosclerosis involves the recruitment of inflammatory cells from the circulation and their transendothelial migration. Several lines of evidence support a crucial role of adhesion molecules in the development of atherosclerosis and plaque instability. Expression of VCAM-1, ICAM-1 and L-selectin has been consistently observed in atherosclerotic plaques (Blankenberg *et al.,* 2003). Blockade of the function or expression of CAM has thus emerged as a new therapeutic target in inflammatory diseases. Different drugs are able to interfere with cell adhesion phenomena. In addition, new anti-adhesion therapeutic approaches (blocking monoclonal antibodies, soluble receptors, synthetic peptides, peptidomimetics, etc. are currently in development.

1.3.4 Cycloxygenase and its involvement in inflammation

Cyclooxygenase (COX) is an enzyme that is responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane). There are three different forms of the Cyclooxygenase identified, which are called COX1, COX2 and COX3. COX1 is constantly active and ubiquitously present, while COX2 is inducible and is active with inflammations only (Williams and DuBois, 1996).

COX2-induced production of prostanoids is often implicated in inflammatory diseases, characterized by edema and tissue injury due to the release of many

inflammatory cytokines and chemotactic factors, prostanoids, leukotrienes, and phospholipase (Arslan and Zingg, 1996, Tanaka *et al.,* 1997). Enhanced COX2-induced synthesis of prostaglandins stimulates cancer cell proliferation (Sheng, H, *et al.,* 2001), promotes angiogenesis (Ben-Av *et al.,* 1996; Tsuji *et al.,* 1998), inhibits apoptosis (Sheng *et al.,* 1998), and increases metastatic potential (Kakiuchi *et al.,* 2002). COX2 is also closely involved in the carcinogenesis process (Tsuji *et al.,* 2001) and is overexpressed in adenocarcinoma in comparison with noncancerous mucosal regions in colon cancers (Sano *et al.,* 1995), lung (Achiwa *et al.,* 1999) and gastric cancers (Murata *et al.,* 1999). COX2 is known to be associated with esophageal, head and neck, breast, lung, prostate, and other cancers, indicating a close involvement of COX2 in tumor progression and other pathological phenotypes in various malignant tumors (Tsuji *et al.,* 1998).

Thus, COX2 inhibitors are a new class of nonsteroidal anti-inflammatory drugs (NSAIDs) (Williams GW, 2005) that selectively block the COX2 enzyme. Blocking this enzyme impedes the production of the chemical messengers (prostaglandins) that cause pain and swelling in arthritic inflammation. Pharmacological inhibition of COX2 can provide relief from the symptoms of inflammation and pain; this is the method of action of well-known drugs such as aspirin and ibuprofen. COX2 plays important role in various liver pathogenesis, including in the development of hepatocellular carcinoma (Hu KQ, 2003). Drugs such as sulindac, which inhibit the COX2 pathway, were found to be beneficial in treatment of human esophageal adenocarcinomas (Chen *et al.,* 2002). Colorectal cancers are one of the major causes of cancer in western countries and COX2 over expression is often associated with it. COX2 is one of the genes transcribed in response to activation of NF-κB and hence inhibition of this transcription factor by curcumin has been found to be involved in downregulation of COX2 and hence beneficial in treatment of colorectal cancers (Plummer *et al.,* 1999). Prostacyclins trigger vasodialation and platelet inhibition via counteracting thromboxane A2 and inhibition of COX2 decreases prostaglandin synthesis disrupting the homeostasis and increasing the cardiovascular risk (Spektor and Fuster, 2004). Thus clinical application of COX2

inhibitors will provide new information as to whether COX2 is a useful molecular target. Nevertheless, more studies are required to better understand and utilize this pathway.

1.3.5 Regulation of inflammation by mitogen activated protein kinase (MAPK)

The Mitogen-activated protein (MAP) kinases are a large family of proline-directed, serine/threonine kinases that require tyrosine and threonine phosphorylation of a TXY motif in the activation loop, for activation through a phosphorylation cascade involving a MAPKKK, MAPKK and MAPK, often referred to as the MAP kinase module. Three separate such modules have been identified, based on the TXY motif of the MAP kinase and the dual-specificity kinases that strictly phosphorylate their specific TXY sequence. They are the extracellular signal regulated kinases (ERKs), c-jun N-terminal kinases (JNKs) and p38 MAPKs. The ERKs are mainly associated with proliferation and differentiation while the JNKs and p38MAP kinases regulate responses to cellular stresses.

1.3.5.1 p38 MAPK: IL-1 and LPS can activate p38 MAPK and hence believed to play a critical role in inflammation. p38 is recognized to be a potent activator of TNF-α. Reports suggests that p38 activity is required for initiation of TNF-α mRNA translation (Lee *et al.*, 1994) and inhibitors of this kinase lead to inhibition of TNF-α mediated damage in subjects of rheumatoid arthritis. SB202190, a prototype of p38 inhibitors is likely to be involved in inflammatory diseases.

1.3.5.2 c-Jun-N-terminal kinase (**JNK):** JNKs are strongly induced in response to pro-inflammatory stimuli and its inhibition has been reported to have beneficial effects in Rheumatoid arthritis (Han Z, 2001). JNKs are involved in the activation of important classes of transcription factors including AP-1, ATF2 which are all involved in the regulation of a wide array of genes involved not only in inflammation, carcinogenesis but also in the normal physiological functioning of the cell. Several different inhibitors of this pathway are produced to control the inflammatory response generated via this pathway.

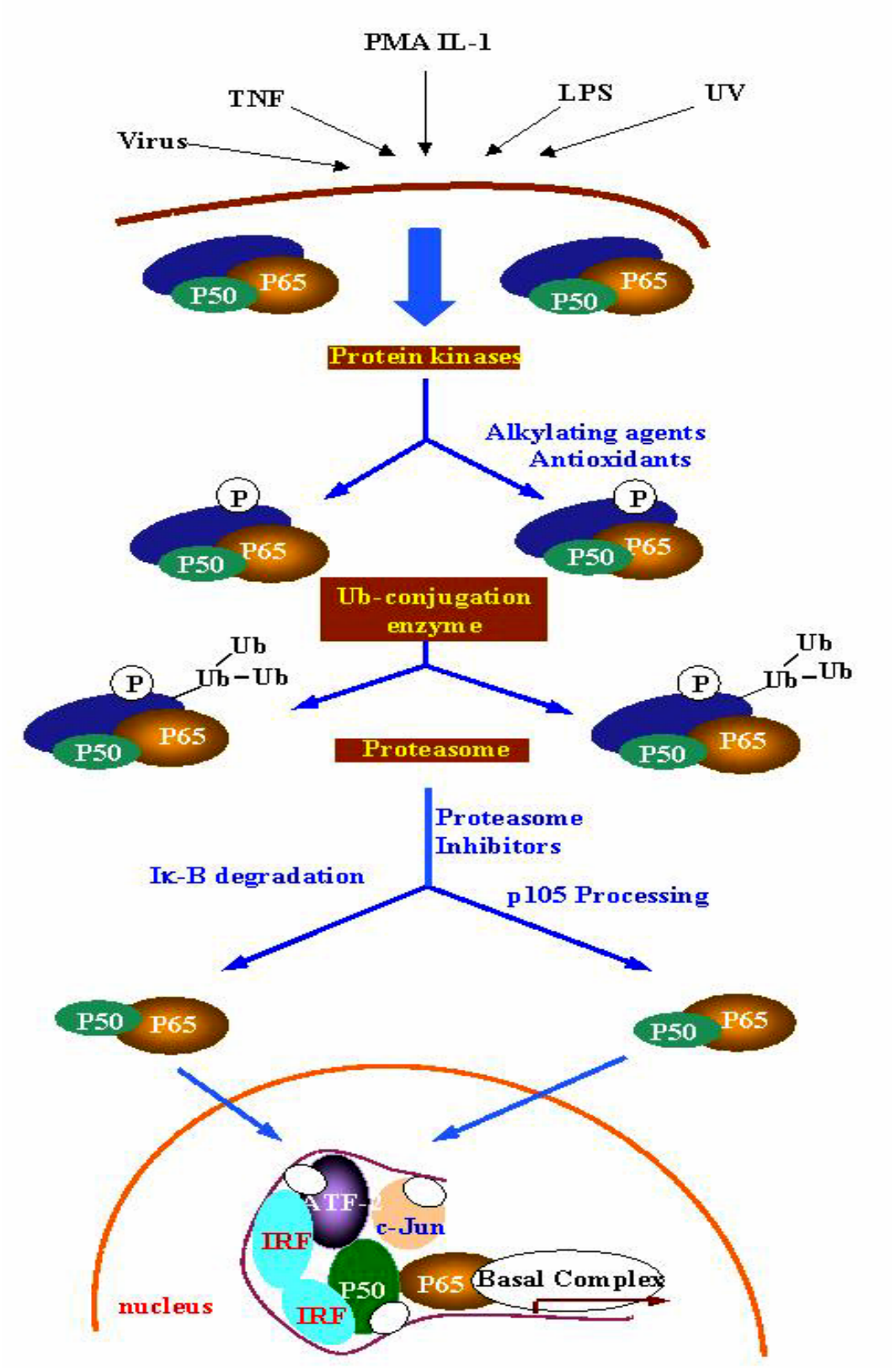
SP6000125 is one such which has been tested in a rat model of Rheumatoid arthritis where it was found effective not only in reducing inflammation (paw swelling) but also in prevention of tissue damage (Favata *et al.,* 1998).

1.3.5.3 Extracellular signal-regulated kinase (ERK): Less is known about the ERK family of MAPKs when compared to the other members. However, a recent publication indicates that the MAP3K Tpl2/Cot is responsible for ERK activation in response to bacterial endotoxin (LPS) in macrophages (Dumitru *et al.,* 2000). The knockout of Tpl2 abolished ERK activation by LPS (Medzhitov R, 2001) and most importantly inhibited the induction of TNF-α release. Since TNF-α is a major mediator of chronic inflammation, the inhibition of ERK offers a new way for therapeutic intervention. Although no direct ERK inhibitors have been reported as yet, several inhibitors that interfere with the activity of the MKKs that act upstream to the ERKs and downstream to the MAP3Ks, MEK1 and MEK2 have been described. These compounds, including PD 98059 (Dudley *et al.*, 1995) and U0126, (Favata *et al.,* 1998) are quite effective inhibitors of ERK activation.

1.3.6 Regulation of inflammation by transcription factor, NF-κB

An organism responds to changes in the environmental conditions by modulating the gene expression with the help of inducible transcription factors. One transcription factor that serves as a key responder to changes in the environment is the NF-κB, an evolutionarily conserved (from drosophila to man) signaling module. Identified just two decades back, in 1986 by Sen and Baltimore (Sen and Baltimore, 1986), this factor has become the interest of study in several leading laboratories of the world because of the significant role it plays in a wide range of cellular functions starting from cell division, growth and differentiation to cell death by virtue of its ability to modulate expression of over 200 immune, growth and inflammatory genes. To date five members of mammalian NF-κB family are recognized which include p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). They exist in unstimulated cells as homo- or heterodimers bound to IκB family proteins. Binding to IκB masks the NLS of NF-κB, preventing the

Various agents involved in activation of NF-κB



translocation of complex to the nucleus. Signaling of the NF-κB pathway occurs by the classical or alternate pathway (Bonizzi and Karin 2004). In classical pathway, cells' encounter with pro-inflammatory signals such as TNF-α or LPS leading to activation of the β-subunit of the IκB kinase (IKK) complex, which then phosphorylates IκB proteins on two N-terminal serine residues (S32, S36). The alternate pathway involves NIK mediated activation of IκK-α leading to phosphorylation and processing of p100, generating p52: RelB heterodimers. The freed NF-κB dimers translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of the target genes. Activated NF-κB can be downregulated through multiple mechanisms including the well-characterized feedback pathway whereby newly synthesized IκB-α protein binds to nuclear NF-κB and exports it out of cytosol. The list of activators of NF-kB is bewildering (Pahl HL, 1999) with some of the well characterized ones being TNF-α, IL-1, ROS, UV-light, ischemia, lipopolysacharide, bacteria, viruses, and several chemical agents. All the above ones are potent inflammatory or carcinogenic agents, eventually indicating the involvement of NF-κB in inflammation and cancer development. NF-κB has been reported to activate several genes including

Growth factors such as G/M CSF, M-CSF, G-CSF involved in the cell development as well as tumour progression,

Cell adhesion molecules such as ICAM-1, VCAM, E-Selectin, P-Selectin, and MMP involved in tumour angiogenesis and metastasis,

Cytokines like TNF-α, IL-1, IL-2, IL-6, IL-8, IFN-γ, all being potential inflammatory agents,

Transcription regulators involved in apoptosis or oncogenesis such as p53, IκB, c-rel, c-myc and

Anti-apoptotic proteins like TRAF-1, TRAF-2, c-IAP1, c-IAP2 (Pahl HL, 1999).

Thus though NF-κB is essential for the normal functional of a cell, an aberrant activation of NF-κB could lead to severe consequences. Hence this transcription factor appears to be an ideal target for controlling several diseases.

1.3.7 Role of reactive oxygen species (ROS) in inflammation:

1.3.7.1 Reactive oxygen species: These include molecules like Hydrogen peroxide (H_2O_2), ions like hypocloride ion ($HOCL^-$), radicals like hydroxyl radical ($^{\cdot}OH$), which is the most reactive of all ROS known to chemistry), superoxide ion (O_2^-) which is both an ion and a radical. ROS are formed by different mechanisms:

a) By interaction of ionizing radiations with biological molecules (Heck *et al.*, 2004).

b) As an inevitable byproduct of cellular respiration from mitochondria.

c) In addition to mitochondria, cytochrome p450 and their reductases, the xanthine/ xanthine oxidases and nitric oxide synthetase also generate ROS.

d) Another abundant source of ROS are the professional phagocytes, which include the neutrophiles and macrophages, which have long been known to express an NADPH oxidase which generate high levels of O_2^-.

Infact, recent recognition of many cells expressing homologues of catalytic subunits of NADPH oxidase, pg 91^{phos}, has raised questions about their mode of regulation to produce ROS either constitutively or in response to cytokines, growth factors and calcium signals. IFN-γ and TNF-α, the major pro-inflammatory cytokines induce cell death via JNK/SAPK pathway, which results in production of ROS and disturb the mitochondrial membrane potential (Kim *et al.*, 2005). Generation of oxidants is thus an unavoidable consequence of normal growth and development. They can also serve a protective function by killing invading bacteria and tumor cells (Babior BM, 1978).

However, an excessive production, which could result either from an exogenous stimulant or an imbalance in the endogenous regulatory processes, could become detrimental. For instance, skin is exposed to various endogenous and environmental pro-oxidant agents that are responsible for allergic and inflammatory skin diseases like atopic dermatitis, utricaria and psoriasis (Okayama Y, 2005). Lung encounters highest degree of oxidant burden and several lung inflammatory states such as asthma, chronic obstructive pulmonary disease (COPD) and parenchymal lung disorders are aggravated upon oxidative stress (Kinnula VL, 2005). COPD status and thus antioxidant therapies may be

helpful in treatment of COPD (Rahman I., 2005). An increased ROS level is reported to be during chronic course of Leprosy and antioxidant therapy along with multidrug therapy is shown to be beneficial (Vijayaraghavan *et al.,* 2005). Even neurodegenerative diseases such as Alzheimer's disease, Parkinson's diseases, Huntington's disease, which are complex and involve many CNS tissue type structures and biochemical processes are shown to be modulated by production of ROS associated inflammatory responses, wherein treatment with anti-inflammatory or antioxidant drugs are postulated to be beneficial (Manton *et al.,* 2004). ROS also contributes to brain damage during bacterial meningitis and interference with ROS is believed to be a novel therapeutic strategy to improve the outcome of meningitis (Pfister *et al.,* 1999) Excessive production of ROS is reported to lead to progression of cancers (Rahman I, 2005). Cancer patients have been detected to undergo higher levels of oxidative stress when compared to normal tissues. Use of antioxidants like Desferal, Tempol and DMSO were found to enhance the killing by anticancer drugs in a model of human Burkitt Lymphoma cells (Shacter *et al.*, 2000).

1.3.7.2 Redox sensitive transcription factors:

Cells' response to a stimuli results in alteration of intracellular signaling pathways, which culminates into transcription of several genes. Transcription factors are the first signaling components identified as redox-sensitive signaling proteins. Important among them are NF-κB and AP-1.

1.3.7.2.1 Redox regulation of NF-κB: Members of NF-κB/Rel family of transcription factors are activated in various inflammatory and oxidative stress responses and regulate genes responsible for these states, including IL-1β, TNF-α, IL-6, IL-8, E-Selectin, VCAM-1, ICAM-1, and GM-CSF. Several reports have shown activation of NF-κB to occur in response to ROS (Schreck *et al.,* 1991). However, this is highly cell type and stimulus specific (Anderson *et al.,* 1994, Hayakawa *et al.,* 2003). Depletion of GSH (body's intracellular antioxidant) or increase in the GSSG level upon oxidative stress has been shown to induce rapid phosphorylation, ubiquitination and degradation of IκB (Ginn-Pease and Whisler, 1996). However, it is not clear if oxidative stress directly

stimulates IKK activity or elevated GSH inhibit IKK activity. Changes in GSH levels have been reported to modulate the proteosome enzymatic activity leading to regulation of NF-κB (Anderson *et al.,* 1994, Ishii *et al.,* 2000). Tyrosine phosphorylation of IκB is supposed to be a mechanism of H_2O_2 induced IκB-α phosphorylation. Inhibition of TNF-α mediated activation of NF-κB and its gene products in presence of NAC support the role of ROS in activation of NF-κB (Harper *et al.,* 2001). However, there are contradictory reports of inhibition of NF-κB induced by TNF- α in presence of NAC to be ROS independent and so it still remains to confirm the exact link between ROS and NF-κB and the mechanism involved in the regulation of each other.

1.3.7.2.2 ROS and its regulation of AP-1:

AP-1 family of transcription factors consists of members of Jun (c-Jun, JunB, JunD), Fos (c-Fos, Fra 1, Fra2), ATF, and MAF proteins which play critical roles in regulation of cell proliferation, differentiation, stress, apoptosis and tumor promotion, and are shown to be redox regulated (Klatt *et al.,* 1999, Macian *et al.,* 2001). The redox regulation occurs mainly in the nucleus (Schenk *et al.,* 1994). However, the activation of AP-1 in the cytosol occurs through oxidative mechanisms by members of MAPK, which phosphorylate the AP-1 leading to its subsequent activation (Wilhelm *et al.,* 1997; Okazaki *et al.,* 1995). GST-pi (Glutathione S-Transferase pi) is reported to regulate AP-1 activity by forming the GST-pi –JNK complex that prevents JNK activation, the upstream kinase responsible for AP-1 subunits activation as maintained by free radical scavengers. Oxidative stress releases this GST-pi-JNK complex and leads to AP-1 activation. However, further studies still needs to be done to track down the mechanism of redox regulation of AP-1.

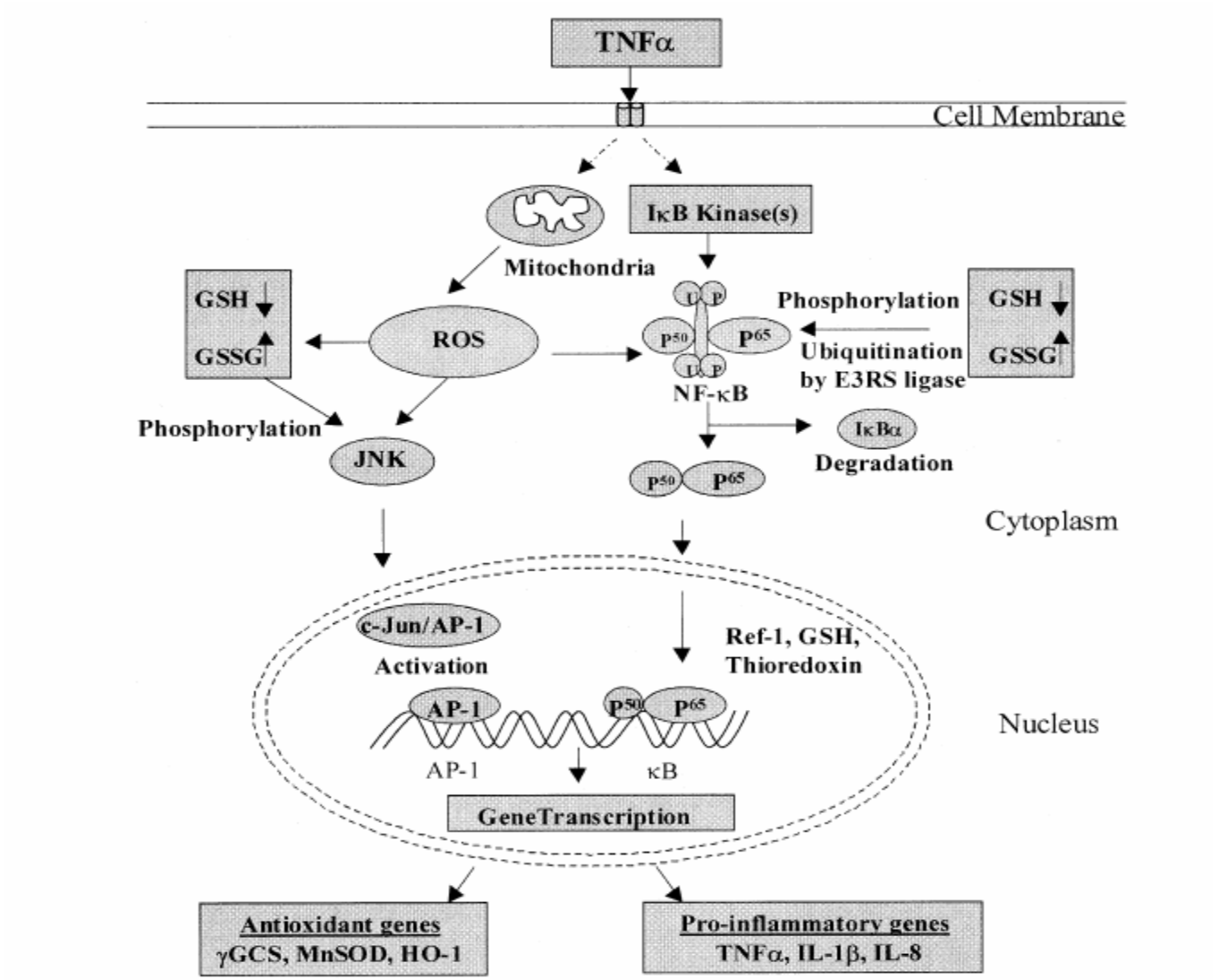
1.3.7.3 Cells endogenous mechanism to combat ROS

1.3.7.3.1 Role of Glutathione

To combat the damage caused due to ROS production, body has developed endogenous anti-oxidant defense system, the critical among which is the redox regulatory

Glutathione (GSH). Reduced glutathione is a ubiquitous, essential tripeptide (L-γ-glutamyl-L-cysteinyl glycine) containing a side chain sulfydryl (-SH) residue that enables it to protect cells against oxidants and electrophilic compounds (Rahman, I, 2000). The level of GSH is found to be altered in several inflammatory disorders including acute and chronic inflammatory pulmonary fibrosis, acute respiratory distress syndrome, cystic fibrosis, asthma and in regular smokers. The redox regulation of GSH is mainly linked to modulate kinases responsible for activation of important inflammation regulating transcription factors like NF-κB and AP-1. Reports have shown that oxidative stress, including lipid peroxidation products or depletion of GSH or increase in GSSG levels leads to rapid phosphorylation, ubiquitination and degradation of IκB complex, thus activating NF-κB (Mercurio and Manning, 1999, Mihm *et al.,* 1995). When subjected to reducing conditions by way of treatment with cystein donor N-acetyl-L-cystein (NAC) serine phosphorylation of IκB following TNF-α treatment is inhibited, leading to down regulation of NF-κB as shown in endothelial cells (Cho *et al.,* 1998). Reports have shown that TNF-α activates NF-κB via an oxidant sensitive pathway and this can be blocked by pretreatment with NAC in macrophages and monocytes like U-937 (Vlahopoulos *et al.,* 1999). Study by Manna et al (Manna *et al.,* 1999) showed that over expression of γ-GCS and GSH levels led to inhibition of NF-κB activated by TNF-α. Work by Rahman and coworkers has demonstrated that exposure to TNF-α resulted in a marked depletion in GSH levels with a subsequent increase in GSSG levels in the cell which led to the activation of NF-κB (Rahman *et al.,* 1999). Also alteration in the GSH/GSSG ratio led to modulation of JNK, which in turn affected the activation status of AP-1 (Hutter *et al.,* 2000; Singh *et al.,* 1998).

Modulation of GSH levels by TNF–α



Model for the mechanism of NF-κB and AP-1 activation leading to gene transcription. TNF-α acts via its receptors on IκB kinase(s) and/or mitochondria to generate ROS, which are involved in the activation of NF-κB. Activation of NF-κB involves the phosphorylation, ubiquitination, and subsequent proteolytic degradation of the inhibitory protein IκB. Free NF-κB then translocates into the nucleus and binds with its consensus sites. The intracellular redox ratio of GSH/GSSG levels and the intranuclear presence of Ref-1, GSH, and thioredoxin can modulate AP-1 and NF-κB activation. Similarly, AP-1 (either c-Jun/c-Jun [homodimer] or c-Fos/c-Jun [heterodimer]) is activated by TNF-α-mediated phosphorylation of the JNK pathway leading to the activation of AP-1, which binds with its TRE consensus region. Activation of NF-κB/AP-1 leads to the co-ordinate expression of protective antioxidant and pro-inflammatory genes.

Thus GSH redox (thiol disulfide) status is critical for various biological events that include modulation of redox regulated signal transduction, transcriptional activation of specific genes, storage and transport of cysteine, regulation of cell proliferation, apoptosis, immune modulation and inflammation (Brown *et al.,* 1994; Droge *et al.,* 1994) and hence agents which could increase the GSH levels can prove to be very beneficial in the treatment of oxidative stress related disorders.

1.4 Endogeneous resoluters of inflammation

Once the leukocytes and their exudates have entered the site and neutralized the inciting agent, they must be eliminated and cleared in a regulated fashion. This acute resolution is a burgeoning area of inflammatory research that might help to understand the etiology of chronic inflammation. Some of the important internal mediators of resolution include….

1.4.1 Resolution by prostacyclins

Prostaglandins (PGs) are the major products derived from a Cycloxygenase (COX) catalyzed reaction in a variety of tissues and cells, including those of immune system such as mast cells, platelets, T cells, and macrophages. Arachadonic acid, liberated from cell surface by action of Phospholipase A2, is metabolized by COX1 or COX2 to PGG2 and then PGH2. PGH2 then serves as a substrate for a series of downstream synthetases to give rise to different prostaglandins.

Among them PGD2 is reported to be involved in inhibition of platelet aggregation, vascular and non vascular smooth muscle relaxation, sleep wake regulation and inflammation (Urade and Hayaishi, 2000). Mice deficient in PGD2 synthetase, the enzyme that synthesizes PGD2 showed an exaggerated inflammatory response which failed to resolve readily. Lymphocytes from these mice showed enhanced cytokine profile and pro-inflammatory NF-κB activity. PGD2 synthase transfected cells showed resistance to bleomycin induced lung injury (Ando *et al.,* 2003). PGD2 undergoes dehydration to yield biologically active PG of the J2 series. These PGJ2 act via both PPARγ dependent

and independent manner resulting in suppression of various pro-inflammatory signaling pathways including NF-κB, AP-1 and STATs, cytokines like IL-1β, TNF-α, IL-12 and NOS in macrophages and dendritic cells (Ricote *et al.,* 1998; Gosset *et al.,* 2003). These PGJ2 preferentially inhibit the monocyte but not the PMNs indicating its role in regulation of the chronicity of inflammation rather than the onset. Reports have also shown that one of the inflammation resolutory activity of PGJ2 and PGD2 to be via apoptosis of the otherwise resistant macrophages. Thus PGJ2 seem to be more involved in preventing complications arising from excessive tissue fibrosis and wound healing. PGJ2 have shown protective role in experimental models of ischemia reperfusion injury, inflammatory bowel disease, adjuvant-induced arthritis, and experimental autoimmune encephalomyelitis.

Thus PGD2 and PGJ2 contribute to the resolution of inflammation through various mechanisms that include inhibition of expression of pro-inflammatory genes, the induction of apoptosis in activated macrophages and myofibroblasts, and the activation of PPARγ. Reports provide support for the 15 d PGD2 and PGD2 as a novel therapeutic agent. However there is a tissue specific role of PGD2 in resolving inflammation.

1.4.2 Involvement of Haemoxygenase in resolution

Haemoxygenase (HO) is a stress inducible enzyme that catalyzes the degradation of haeme to liberate free iron, CO, Biliverdin and Bilirubin (Ryter *et al.,* 2002). It is recognized as to be playing crucial role in resolution of acute pleuritis (Willis *et al.,* 1996). HO-1 is reported to inhibit the PMN trafficking and thus help in resolution of onset of acute inflammation. HO-1 plays important role in controlling oxidative insults through the anti-oxidant properties of Bilirubin and Biliverdin. Upregulation of HO-1 with anti-CD14 protects mouse allograft against transplantation rejection (Hancock *et al.,* 1998). 15 PGJ2 are believed to be exerting their effects via generation of HO-1. Thus compounds, which regulate HO-1, might represent another novel strategy to control inflammation.

1.4.3 Role of glucocorticoids and annexin in resolution

Glucocorticoids are endogenous potent anti-inflammatory mediators released within minutes of sensing stress or tissue injury and serve as important regulators of inflammation. Removal of this primary break leads to augmentation and prolonged inflammation (Munck *et al.,* 1984). They help in enhancing the resolution process probably by selectively involving eosinophil apoptosis and by enhancing macrophage phagocytosis of apoptotic bodies. This thus signifies the role of steroids in treatment of inflammation like allergies wherein these steroids reprogramme the ongoing inflammatory lesion down a pro-resolution pathway. The unbound glucocorticoid receptor (GR) remains in the cytosol in an inactive form bound to regulatory proteins such as heat shock proteins 70, 90; p59 immunophilin and phosphoprotein p23 (Adcock and Ito, 2000). Upon binding to the glucocorticoid, the glucocorticoid/ GR complex undergoes a conformational change leading to dissociation from the regulatory protein. Now this complex can exert two known functions. It could either translocate to the nucleus, the most well studied genomic function of glucocorticoids, to drive the transcription of specific genes, or it could also exert its non-genomic action which involves regulation of transcription factors like NF-κB, AP-1, the mechanism involved is however, not well studied. Nevertheless, glucocorticoids seem to serve as potent anti-inflammatory agents.

Glucocorticoidds also modulate the expression and release of regulatory proteins like annexin-1, which possess anti-inflammatory properties. Annexin-1 is shown to act via the LXA4 receptors (Perretti *et al.,* 2002) indicating that this receptor is evolutionary conserved to modulate the inflammatory response. Passive immunization of mice with neutralizing anti- annexin-1 sera led to prolonged PMN recruitment, alteration of cytokine profile and release of proteolytic enzymes (Perretti *et al.,* 1996). Annexin-1 null mice also showed aggregation of the inflammatory mediators highlighting the potential therapeutic application of annexin-1 mimetics.

1.4.4 Resolution by Lipoxins and Resolvins

1.4.4.1 Lipoxins (LX)

These are a family of icosanoid metabolites produced by transcellular metabolism of Arachadonic acid by LOX/LOX (Lipoxygenase). They are produced by action of 5LO in macrophages and neutrophiles on the 15-(S)-hydroxyicosatetranoic acid released by epithelial cells (Chavis *et al.,* 1992) or by action of 12-LO (lipoxygenase) on macrophage (Serhan and Sheppard, 1990) or 15-LO on leukotriene A4 released from neutrophiles (Levy BD, *et al.,* 1993). Several lines of evidence suggest that LXs are potent anti-inflammatory agents. LX stimulates a) phagocytosis of apoptotic neutrophils by macrophages to resolve inflammation (Godson C, *et al.,* 2000), b) LXA4 inhibits neutrophil transmigration across epithelial cells and endothelial monolayer (Papayianni *et al.,*1996), c) inhibits TNF-α induced IL-1β released by PMN (Hachicha *et al.,* 1999), d) decreases LTB4 released by human neutrophiles (Vachier *et al.,* 2002), e) inhibits IL-1β induced IL-6 and IL-8 release in human synovial fibroblasts (Sodin-Semrl *et al.,* 2000). LXA4 was identified along with PGE2 in a mouse model of allergic oedema during the clearance of the lesion, inhibition of which led to prolongation of the oedema. LX act via G-protein coupled receptor ALXR which are detected on the surface of monocytes, macrophages and neutrophiles (Maddox *et al.,* 1997). Receptor bound LXA4 activates in myeloid cells phospholipase A2 and phospholipase D (15). LX and their analogs are highly effective in treatment of a range of skin diseases (Schottelius *et al.,* 2002), gastritis (Souza *et al.,* 2003).

1.4.4.2 Resolvins:

The omega 3-fatty acids (constituents of fish oils) - DHA (docosahexaenoic acid) and EPA (eicosahexaenoic acid), which have been recently shown to be metabolized to products possessing anti-inflammatory properties and are aptly termed as Resolvins (Serhan *et al.,* 2000). These were found to be released during the resolving phase of aspirin treated TNF-α induced inflammation. In endothelial cells expressing COX2 and treated with aspirin convert EPA to 18R- hydroxyeicosapentaenoic acid (HEPE) and 15R-

HEPE, both of which are taken up by the PMNs to generate classes of novel trihydroxy mediators that potently inhibit neutrophil transmigration. They have been found to inhibit microglial cell cytokine expression and ameliorate experimental models of dermal inflammation and leukocyte accumulation in peritonitis at even nanogram doses (Serhan *et al.,* 2002). Thus collectively, LX and Resolvins represent novel class of anti-inflammatory agents involved in resolution of acute inflammation and implicated in diseases including artherosclerosis, peridontitis, chronic liver diseases and asthma (Van Dyke *et al.,* 2003; Claria *et al.,* 1998; Levy 2002).

1.4.5 Role of NF-κB (p50) in resolution of inflammation

NF-κB is one of the well-studied transcription factors that play a pivotal role in inflammation by regulating the expression of several pro-inflammatory cytokines, chemokines and inducible enzymes (Karin M, 1998a). Several inhibitors of this pathway are documented to have potential role in resolution of inflammation. However, in contrast to pharmacologically manipulating this pathway, recently NF-κB has also proved to possess anti-inflammatory properties that mediate the resolution of acute experimental pleuritis (Lawrence *et al.,* 2001), This involves the recruitment of alternate DNA-binding complexes such as p50-p50 homodimers which might antagonize NF-κB dependent pro-inflammatory gene expression while simultaneously promoting the expression of genes that are required for the resolution of inflammation. This promotes the leukocyte apoptosis through recruitment of NF-κB DNA-binding complexes that lack transcriptional activation domains and act as dominant negative inhibitors of anti-apoptotic gene expression. p50-p50 homodimers are found to be persistent during the resolution of inflammation with the subsequent repression of pro-inflammatory gene transcription (Bohuslav *et al.,* 1998). Inhibition of p105 processing to p50 using proteosome inhibitor MG132 led to reduced expression of pro-apoptotic genes BAX and p53 in leukocytes during the resolution of acute inflammation. BAX and p53 are involved in resolution by inducing apoptosis and the expression of these two genes has been found to be regulated

by the p50-p50 complex. Thus, a better understanding of this pathway will lead to the development of drugs to resolve inflammation in a cells' own natural mechanism.

1.4.6 Significance of melanocyte stimulating hormone (MSH) in inflammatory resolution

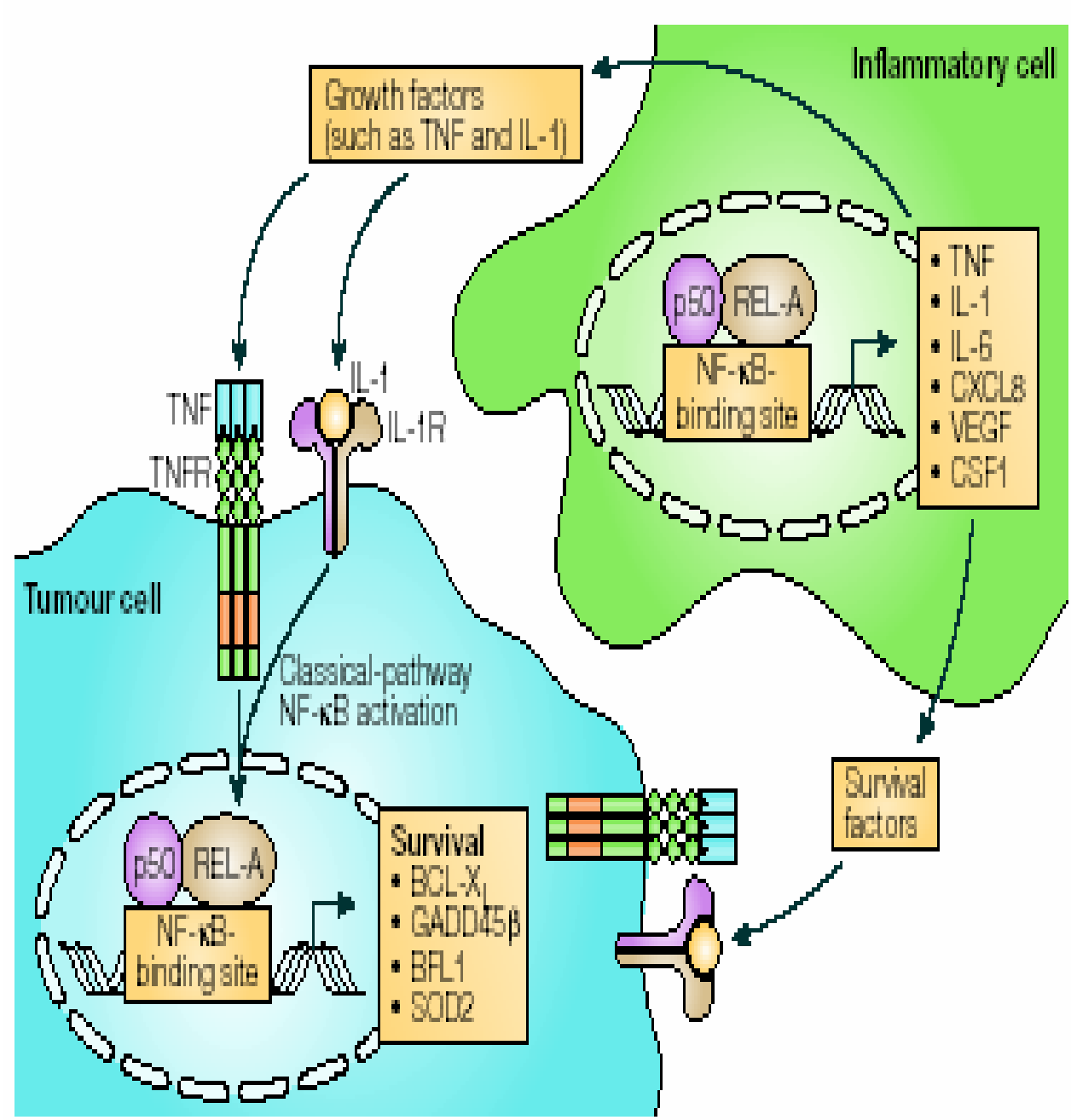
Another family of endogenous resoluters represented by five G-protein coupled receptors is the MSH family of peptides. Originally named for its pigment modulatory effects on the skin, MSH has now come to be recognized as one of the potent anti-inflammatory peptides produced by our body. The hormone is a tridecapeptide (α-MSH [1–13] SYSMEHFRWGKPV) that is synthesized by the posttranslational processing of the POMC peptides produced by the pituitary gland (Benjannet *et al.,* 1991). However MSH has also been reported to be synthesized by several other cells of the body including the monocytes, macrophages, neutrophiles, epithelial cells, keratinocytes, endothelial cells (Rajora *et al.,* 1996; Wong *et al.,* 1997; Luger *et al.,* 1993) all giving an indication that this small peptide probably does more than its well known pigment regulatory functions. MSH act through G-protein coupled receptors. Five families are known (MC-R 1to5) which differs slightly in their tissue distribution and function. The physiological functions of the hormone involve regulation of food intake, weight homeostasis, cardiovascular functions, thermoregulation, and skin and hair pigmentation. In comparison to other receptors, the MC-R1 is well documented on the cells of the immune system indicating its role in the MSH mediated immune system modulation. The plasma concentration of α-MSH in a large population of 234 normal blood donors was 21.30+-0.63 pg ml- with no difference between men and women. (Catania, A.1998) and this concentration is remarkably constant over time in the normal patients. The synovial fluid of patients with arthritis showed a significant rise in the circulating α-MSH levels, which must be the results of local release of the peptide by the circulating monocytes and macrophages at the site. Increased circulating α-MSH was also found in patients with acute myocardial infaraction who receive thrombolytic therapy (Airaghi *et al.* 1995), in multiple sclerosis patients with high disability score and in patients on chronic hemodialysis with detectable

plasma endotoxin (Airaghi *et al.,* 2000). All this suggests the presence of high levels of α-MSH in inflammation and hence it is reasonable to believe that α-MSH plays a role in counteracting the action of inflammatory mediators. The septal regions of the brain which regulate the temperature have been shown to express MC-3 and MC-4 R. Administration of α-MSH externally at doses which do not influence the normal body temperature, reduce the fever caused by pyrogens much more effectively than even the standard acetaminophen (Murphy *et al.,* 1983). Corticosteroids have been found to raise the level of α-MSH (Catania *et al.,* 1991). The explanation for this goes as, the corticosteroids are produced as a host's internal response to the pyrogen, which in turn facilitates the release of α-MSH from the pituitary. Overall, the research indicates that α-MSH is also an effective anti-pyretic agent. α-MSH also is reported to possess anti-bacterial and anti-fungal properties as it was found to inhibit the growth of *Staphylococcal aureus* and *Candida albicans* (Cutuli *et al.,* 2000).The hormone has already proved its efficacy in the treatment of several inflammatory disorders including rheumatoid arthritis (Ceriani *et al.* 1994), adult respiratory distress syndrome (Guarini *et al.* 1997), respiratory arrest, inflammatory bowel disease (Rajora *et al.* 1997), however the mechanism of action is still not well elucidated. With the well documented anti-pyretic and anti-inflammatory actions with no known side effects it certainly becomes interesting to study further the biological significance of presence of MSH receptors on different cells, how it modulates the functioning of these cells in response to a injurious signal and the mechanism involved in regulation of such signals. Such a study would definitely aid in designing therapeutics based on MSH and its related peptides.

1.5 Link between cancer and inflammation: NF-κB

Inflammation as discussed in the earlier sections involves the interplay of several genes that include cytokines like TNF-α, IL-1, IL-6, IL-8, IL-12, adhesion molecules like ICAM-1, VCAM and other factors like MMP-9, MIP-1α, Growth factors. Many of these factors are also documented during tumor development, angiogenesis and metastasis. Hence for several years researchers speculated inflammation to be responsible for cancer

Cross-talk between Inflammatory cells and tumor cells



development. There are also reports of these groups of factors being the target genes of activated NF-κB. Therefore, there appears to be a link between Inflammation - NF-κB - Cancers. This link has reported recently by Michael Karin's group using some elegant experiments in which they manipulated the levels of NF-κB in the tumors and its microenvironment. They examined the IKK-β mediated inflammation and tumorigenesis in a mouse model of colitis-associated cancer (CAC). Deletion of IKK-β led to a decrease in the incidence and development of the CAC in both the enterocytes and myeloid cells tested but via different mechanisms. In the enterocytes it seemed to be involved in the early stages of tumor i.e. in the tumor initiation and/or promotion while in the myeloid cells IKK-β appeared to up regulate the factors responsible for tumor growth (Greten et al., 2004). In another report, the activation of NF-κB was demonstrated to produce inflammation leading to tumor through expression of TNF-α, while inhibition of this resulted in tumor regression via the TRAIL (Luo *et al.*, 2004). Thus, inflammation and tumorigenesis seemed to be closely linked, with NF-κB sandwiching them together. Several pharmaceutical companies are developing drugs to inhibit NF-κB to combat cancer, which should be tested carefully to understand if it actually is able to attack tumorigenesis, as this would depend on the cell type and surrounding environment also. Some of the drugs, which serve as anti-inflammatory agents by inhibiting NF-κB, are also, turning out to be having anti-tumour activities, for example aspirin. Hence there is demand for inhibitors of NF-κB with least side effects like the plant-derived polyphenols, which would help in prevention and also treatment of cancer.

1.6 Anti-cancer and anti-inflammatory therapy

Chemotherapy is widely and successfully used in the treatment of several types of cancers. However, resistance to these is a major drawback, which impedes the effective usage. While many of the chemotherapeutic drugs work by activating tumor suppressor proteins like p53, they also lead to activation of NF-κB. For example, taxanes, Vinca alkaloids and Topoisomerase inhibitors used as anti-cancer agents, activate NF-κB.

Topoisomerase II inhibitor, Doxorubicin activates NF-κB by stimulating IKK activity (Bottero *et al.*, 2001). Vinblastin, a microtubule polymerization inhibitor, enhances the enhancer activity of NF-κB (Bourgarel-Rey *et al.*, 2001). Once activated, NF-κB gives the cell a proliferative signal thereby decreasing the efficacy of these drugs as anti-cancer agents.

This transcription factor is also involved in different inflammatory disorders. Among the anti-inflammatory drugs, Steroids and Non-Steroidal drugs (NSAIDS) are currently in use for treating acute and chronic inflammation. The NSAIDS mainly function as inhibitors of the COX2, the inducer of prostaglandins. PGE2 is reported to induce MMP-2, MMP-9, which trigger the expression of TGF-α, EGF responsible for gastric, epithelial and colonic cancers. The well known NSAIDS, Aspirin and Sulindac that are used as effective anti-inflammatory drugs are shown to regress adenomas in patients suffering from familial adenomatous cancers. The mechanism responsible for this is inhibition of the IKK activity (Yamamoto *et al.*, 1999). Thus, NF-κB appears to be the central target in the treatment of both cancer and inflammation. However, most of these synthetic drugs suffer from lot of side effects. For example, Vioxx and Celebrex that are extensively used in treatment of arthritis were recently withdrawn due to their cardiovascular effects. Hence, a thorough study of the long-term effects of the drugs is needed before releasing them into the market.

In such a scenario, drugs based on endogenous mediators that are inherent to resolution like α-MSH, might represent a new strategy in anti-inflammatory and anti-cancer therapy.

1.6.1 Significance of plant based products

Plant products or drugs based on plant derivatives are gaining considerable attention due to the absence of any reported side effects. Many of the natural products also act via inhibition of the NF-κB pathway. For instance, Genistein, an isoflavonoid from soybean inhibits NF-κB activity via AKT pathway. Resveratol, a polyphenol derived from grapes inhibits the IKK activity (Estrov *et al.*, 2003). Curcumin is another extensively studied

plant derivative exhibiting potential anti-inflammatory and anti-cancer properties, also inhibits the IKK activity (Aggarwal *et al.,* 2004). Lycopene, a carotenoid in tomatoes directly suppresses the p65 nuclear translocation (Kim *et al.,* 2004). Thus by blocking the NF-κB activity, these drugs seem to be potential anti-inflammatory agents. At the same time, they could be used in potentiating the anti-cancer activity of other well known chemotherapeutic drugs. For instance, Cisplatin and Docetaxel, the well known chemotherapeutic agents, activate NF-κB leading to problem of drug resistance. A combination of Genistein with any of these drugs is reported to enhance the chemotherapeutic potential of these drugs in pancreatic cancers (Li, Y, *et al.,* 2004). Hence, plant based natural products alone or in combination with standard chemotherapeutic drugs appears to be a very effective and novel strategy. However, understanding the signaling mechanism of any putative drug to be released is very essential to make the best use of it.

1.7 Importance of Mangiferin

Mangiferin, 1, 3, 6, 7-tetrahydroxyxanthone-C2-beta-D-glucoside, is one of xanthone derivatives and C-glucosylxanthones, is widely distributed in higher plants and is one of constituents of folk medicines. It was first isolated from the bark of the mango tree (Mangifera indica L., Anacardiaceae) way back in the early 1960s and Ramanathan and sheshadri assigned the structure to it. The Mango sap is toxic, causing a rash similar to poison ivy on the skin. The active principals causing sap toxicity are named for mango: mangiferin, mangfiferic acid, and mangiferol. The bark of the tree is an astringent; it is used in diphtheria and rheumatism; it is believed to possess a tonic action on the mucous membrane. In the Philippines, the mangos leaves are prepared as a tea, and the juice of the leaf is considered to be useful in bleeding dysentery (Quisumbing E, 1978). In the traditional Indian system of medicine, mangiferin is also used for melancholia and nervous debility (Bhattacharya *et al.,* 1972). Furthermore, one of traditional Chinese medicines, sann-Joongkuey- jian-tang, also includes the mangiferin (Lin *et al.,* 1996). The leaves are given in the treatment of burns, scalds and diabetes. Mangiferin from the leaves

has been reported to possess anti-inflammatory (Garcia *et al.,* 2002), anti-cancer (Yoshimi *et al.,* 2001), diuretic, chloretic and cardiotonic activities and displays a high anti-bacterial activity against gram-positive bacteria. It has been recommended as a drug in preventing dental plaques. Mangiferin showed anti-viral effects against Herpes Simplex Virus (HSV-1) and HIV (Zheng and Lu 1990; Guha *et al.,* 1996). The antiviral effect of mangiferin and iso-mangiferin was presumably due to their capability of inhibiting virus replication within cells. Mangiferin is also reported to possess potent anti-oxidant property (Sanchez *et al.,* 2000), anti-diabetic (Ichiki *et al.,* 1998; Miura *et al.,* 2001) and also anti-bone resorbtion activity (Li *et al.,* 1998). It is also being used in Cuba to treat patients with inflammatory diseases. Thus mangiferin, with no known side effects seems to possess important therapeutic potential.

1.8 Objective of the present study

Although α-MSH and Mangiferin have come to be recognized for their anti-inflammatory actions, their molecular action still remains to be elucidated. Hence in this scenario, the aim of the present study was to

A) Decipher the molecular signal transduction pathway(s) mediated by α-Melanocyte Stimulating Hormone in different cells participating in the immune response.
    1. Mast cells
    2. Macrophages
    3. Neutrophils
B) Understand the molecular mechanism(s) of action of the plant polyphenol, Mangiferin.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials:

2.1.1 Chemicals:

Arabinoside cytosine (AraC), acrylamide, bis-acrylamide, bromophenol blue, bovine serum albumin (BSA), bestatin, L-buthionine sulfoximine (BSO), glycine, lipopolysacharide (LPS, Escherichia coli 055:B5), calyculin A, catalase, and anticatalase antibody, iodogen, α-melanocyte stimulating hormone (α-MSH), MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], vincristine, taxol, cis-platin, etoposide, adriamycin, doxorubicin, lactacystin, thiobarbituric acid, mangiferin, pyrollidone dithiocarbamate (PDTC), 3-amino-1,2,4-triazole (ATZ), tocopherol, *N*-acetyl cysteine (NAC), sulfosalisilic acid, NADPH, DTNB [5,5-dithiobis(2-nitrobenzoic acid], glutathione reductase, 2-vinylpiridine, triethanolamine, 4-methyl umbelliferyl phosphate, FMLP, nitro blue tetrazolium (NBT), p-nitrophenyl L-D-glucuronide, melanocyte growth stimulatory activity (MGSA), Histopaque, dextran, ortho-phenylenediamine (OPD), *p*-nitrophenyl phosphate, *p*-nitrophenyl β-D-glucuronide, N-methoxysuccinyl-Ala-Ala-Pro-Met *p*-nitroanilide, monodansyl cadaverine (MDC), leupeptin, PMSF, TPCK, TLCK, polymixin B-sulphate, sodium molybdate, giesma stain and zinc chloride were obtained from **Sigma,** St. Louis, MO,USA.

Adenosine 3′, 5′-cyclic Phosphorothioate (Rp-cAMPS) and H-8 [(methylamino) ethyl-5-isoquinolinesulfonamide, HCl], human neutrophil elastase, and N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone were obtained from **Calbiochem** (SanDiego, CA, USA).

Dideoxyadenosine (ddAdo) and dibutyryl cAMP were obtained from **LC Laboratory** (San Diego, CA, USA).

Recombinant TNFα, recombinant human IL-8, IL-13, IL-4, IL-1α, TRAIL, and TNF were obtained from **PeproTech Inc**., Rocky Hill, NJ, USA.

Alexa-Fluor conjugated IgG, mounting medium with DAPI, goat anti-rabbit IgG conjugated with fluorescein isothiocyanate, Live and dead cell assay kit and Di-hydrorhodamine were obtained from **Molecular Probes**, Eugene, OR, USA.

RPMI-1640 medium, DMEM medium, phosphate free DMEM, Opti-MEM medium, trypsin-EDTA, antibiotic-antimycotic, freezing medium, fungizone, L-glutamine, fetal

bovine serum (FBS) and TRIzol were obtained from **Gibco BRL** (Gaithersburg, Maryland, USA).

M-MuLV reverse transcriptase, DNA-polymerase, RNase inhibitor, dNTPs and $MgCl_2$ were obtained from **Invitrogen Corporation** (Carlsbad, CA).

The superfect transfection reagent was purchased from **Qiagen** (Hilden, Germany). Human IL-8 assay kit was purchased from **R&D System** (Minneapolis, MN, USA).The plasmid constructs for NF-κB-SEAP, Cox-2-Luciferase, TNFR1, TRADD, TRAF2, NIK, IKK, p65, and dominant negative IκB-α were kindly supplied by Prof. B. B. Aggarwal of the University of Texas M. D. Anderson Cancer Center (Houston, TX).

The pET 41b was obtained from Novagen, Germany

2.1.2 Antibodies :

Anti-IκBα, p65, p50, Cyclin D1, c-Rel, Rel B, IKK, L-8, IL-8R1, IL-8R2, JNK, Melanocortin receptor (MC-1R, MC-2 R, MC-3 R, MC-4R), proteinase 3, cathepsin G, ICAM-1, IL-8R1 (CXCR1), and IL-8R2 (CXCR2), iNOS, COX2, IL-1R, TNFR1, TNFR2, and CD14, ERK1/2, tubulin and actin used were obtained from **Santa Cruz Biotechnology, Inc**., Santa Cruz, CA, USA.

Anti-Poly (ADP-ribose) polymerase (PARP), caspase-3, and caspase-8 were obtained from **PharMingen** (Canada).

Antibody against phospho-IκB-α and phospho-p65 was obtained from **Cell Signaling Technologies,** Beverly, MA, USA..

2.2. Cell Lines:

Human origin:

A375 (human melanoma), HeLa (cervical epithelial cells), Jurkat (T cells), Daudi (B-cells), IRB3 AN27 (murine mesencephalic cells), MCF-7 (breast epithelial cells), HuT-78 (T cells), U937 (histiocytic lymphoma), THP-1 (monocytic macrophages) and HL-60 (monocytic cells) were obtained from American Type Culture Collection. THP-1 and HL-60 cells were differentiated into macrophages (using 10 nM PMA for 16 h) and neutrophils (using 1.3% DMSO in 2 days), respectively.

γ-GCS-overexpressed cells were a gift from Professor Bharat B Agarwal, MD Anderson Cancer Centre, Houston, Texas, USA.

2.3 Buffers and Media:

Whole cell lysis buffer:

20 mM HEPES (pH 7.9)

250 mM NaCl

1% NP-40

2 mM EDTA

1 mM DTT

To be added just before use

2 µg/ml leupeptin

1 µg/ml Aprotinin

0.5 µg/ml benzamidine

SDS-PAGE:

Stacking Gel Mix (10ml):

2.5 ml of 0.5 M Tris-Cl (pH 6.8)

1.66 ml of 30% acrylamide ; bisacrylamide (29:1) Mix

100 µl of 10% SDS

5.63 ml of water

100 µl of 10% APS

10 µl of TEMED.

9% Resolving Gel Mix (10ml):

2.5 ml of 1.5 M Tris-Cl (pH 6.8)

3 ml of 30% acrylamide; bisacrylamide (29:1) mix

100 µl of 10% SDS

4.29 ml of water

100 µl of 10% APS

10 µl of TEMED.

2X SDS loading Buffer:

130 mM Tris-Cl (pH 8.0)

20% (v/v) Glycerol

4.6% (w/v) SDS

0.02% Bromophenol blue

2% DTT

10X SDS-PAGE Running Buffer:

0.25 M Tris-Cl (pH 8.0)

1.92 M Glycine

1% SDS

Western Blot:

10X Blotting Buffer:

0.25 M Tris-Cl (pH 8.0)

1.92 M Glycine

1% SDS

1X Blotting Buffer (2Litres):

400 ml of methanol

200 ml of 10X blotting buffer

1400 ml of water.

PBS:

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

HEPES Buffer Saline:

20 mM HEPES (pH 7.5)

150 mM NaCl

Blocking Buffer:

5% Fat free milk or 2% BSA

0.05% Tween 20

Make up volume with PBS.

Stripping Buffer:

100 mM β-mercaptoethanol

2% (w/v) SDS

62.5 mM Tris-HCl (pH 6.7)

Electrophoretic Mobility Shift Assay (EMSA):

Cytoplasmic Extraction Buffer:

1 M HEPES (pH 7.9)

2 M KCl

0.1 M EGTA (pH 7)

0.5 M EDTA (pH 8)

Nuclear Extraction Buffer:

1 M HEPES (pH 7.9)

5 M NaCl

0.1 M EGTA (pH 7)

0.5 M EDTA (pH 8)

6X EMSA sample loading dye [10 ml]:

25 mg of Xylene Cyanol FF

25 mg of Bromophenol Blue

7.0 ml of water

3.0 ml of Glycerol

10X EMSA binding buffer:

200 mM HEPES (pH 7.9)

4 mM EDTA (pH 8.0)

40 mM DTT

50% glycerol

5X EMSA buffer:

0.25 M Tris-Cl (pH 8.0)

2.0 M glycine

0.01 M EDTA (pH 8.5)

pH made upto 8.5

EMSA Gel Mix (50 ml):

28.66 ml of water

10 ml of 5X EMSA buffer

11 ml of 30% acrylamide: bisacrylamide (29:1) mix

400 µl of 10% APS

40 µl of TEMED

Kinase assay:

Assay buffer (10X):

20 mM HEPES (pH 7.4)

10 mM $MgCl_2$

1 mM DTT

Wash buffer:

20 mM HEPES (pH 7.4)

25 mM NaCl

1 mM DTT

Luria broth:

10 g Tryptone

10 g NaCl

5 g Yeast extract

TB buffer for preparation of competent cells:

10 mM PIPES (free acid)

15 mM $CaCl_2.2H_2O$

250 mM KCl

55 mM $MnCl_2.4H_2O$

Genomic DNA isolation by silica method:

Lysis buffer:

4 M guanidine thiocyanate

1% N-lauryl sarcosyl

10 mM DTT

Wash buffer:

70% ethanol

10 mM EDTA

10 mM Tris-Cl (pH 8.0)

Elution buffer:

10 mM Tris-Cl (pH 8.0)

0.1 mM EDTA (pH 8.8)

50X TAE (1 liter):

242 g of Tris base
57.1 ml of glacial acetic acid
100 ml of 0.5 M EDTA

2.4 Experimental Procedures:

Isolation of PBMCs and neutrophils from human blood.

Neutrophils were separated from fresh peripheral human blood of normal healthy donors by dextran T-500 sedimentation followed by ficoll-paque (Histopaque-1077) density gradient centrifugation method. The oxalated blood was immediately incubated with 3% dextran solution in saline with 1:1 ratio at 37°C for 30 minutes. The erythrocytes (RBCs) form rouleaux and were sedimented at 400xg for 30 minutes at room temperature. The PBMC layer formed above the ficoll level was removed by aspiration and suspended in phosphate buffer saline later to be centrifuged and the pellet suspended in RPMI medium for culture. After removal of the ficoll paque the granulocyte enriched pellet was washed with D-PBS and RBC contaminants in neutrophils were lysed by addition of 0.2% NaCl solution. Then, 1.6% NaCl was added to bring the correct osmolarity of the cell suspension. After centrifugation at low speed for 5 minutes, the pellet was taken and suspended in medium. The purity of the separated cells was examined by staining the cells with giesma stain and observed under phase contrast microscope. The isolated cells were up to 94-96% neutrophils. The viability of the cells was checked with trypan blue exclusion test, wherein, the viable cells exclude the dye and the non-viable cells take-up the dye giving a blue coloration. The diluted cell suspension was taken in 0.2% trypan blue dye in saline and dyes positive and negative cells were counted separately under microscope using Hemocytometer, which were expressed in percentage. The cells were found to be 98% viable by trypan blue dye exclusion test.

Neutrophils and macrophages differentiation and isolation:

THP1 cells were stimulated with 10 ng/ml PMA for 16 hours and then adherent cells were used as macrophages (Sarkar *et al*., 2003). HL-60 cell line was maintained in complete medium of RPMI-1640. These cells were cultured in presence of 1.3% DMSO for 2 days. These cells were differentiated into neutrophils (Kanayasu-Toyoda *et al*., 1999, 2003). These cells were well characterized as neutrophils by detecting the level of IL-8R expression, morphology, IL -8 induced chemotaxis, and

PMA-induced oxidative burst response and enzyme release compared to normal HL-60 cells. Hence these cells were considered as neutrophils for further studies.

Radiolabeling of IL-8 and other ligands:

Interleukin-8 was iodinated by Chloramine T following the method of Grob *et al.*, with slight modification. The $Na^{125}I$ was mixed with 50 µl of 100 mM phosphate buffer (pH 7.4) in a 1.5ml polypropylene tube. After gentle mixing the tubes were kept at 23°C water bath. 10 µg IL-8 was then added with this mixture. The idonated reaction was started by addition of 30 µl of freshly prepared Chloromine T (1 mM) solution. After proper mixing, the reaction was continued for 60 seconds and then the reaction was terminated by addition of 30 µl of 57.7 mM sodium metabisulfide. Next, 190 µl D-PBS containing 5 mg/ml BSA was added to the reaction mixture. The entire mixture was transferred to a sephadex G-10 column (20cm x1cm) equilibrated with D-PBS containing 5 mg/ml BSA for separation of free iodine. The iodinated protein was eluted by addition of 800µl-equilibrated buffer in each fraction. About 16-column fraction were collected and the count was taken in each fraction in Gamma counter. The first peak, obtained from the counts was due to labeled protein and the second peak for free iodine. The labeled protein (IL-8) containing fraction was aliquoted and kept at -20°C.The specific activity of labeled IL-8 was $1x10^7$ to $4x10^7$ cpm/µg of the protein. The anti-rabit IgG was $3x10^7$ to $5x10^7$ cpm/µg protein.

Receptor binding Assay:

Freshly prepared human neutrophils were suspended in RPMI-1640 (10^7 cells/ml) containing 20 mM HEPES buffer pH 7.2, 5 mg/ml BSA. Cell suspension (200 µl) was taken in each tube for subsequent experiments. After incubation under different condition the cells were cooled on ice. Radiolabelled IL-8 binding was carried out at 4°C for 2 hours using ^{125}I IL-8 (4ng/tube). Then the cells were centrifuged at 4°C for 20 seconds at 10,000xg. The supernatant discarded and the cell pellet was resuspended in ice-cold medium and layered onto ice-cold 800 µl of 10% sucrose in D-PBS and centrifuged at 4°C for 2 minutes at 10,000xg. Only neutrophils passed

through the sucrose solution and unbound ^{125}I IL-8 remained above sucrose solution. The supernatant was removed carefully and the pellet containing part of the tube was taken. The radiolabelled ligand bound to the cell was measured in Gamma counter (Grob *et al.*,).

Enzyme release assay:

Neutrophils/Macrophages (1 x 10^7 cells/ml) were suspended in D-PBS containing glucose (1 mg/ml) and BSA (5 mg/ml). Cells were treated with different modulator (s) and then washed and suspended in fresh medium. Cytochalasin B (5 µg/ml) was added for 5 minutes at 37°C. The supernatant was taken for different enzyme assay.

Myeloperoxidase: Myeloperoxidase activity was measured taking the 25 µl supernatant and 100 µl substrate solution of orthophenylenediamine (OPD) (0.75 mg/ml) and H_2O_2 (0.01%) in citrate phosphate buffer (0.1 M, pH5.2). After 30 minutes at room temperature, the reaction was stopped with addition of 50 µl of 4 N H_2SO_4 and absorbance was measured at 492 nm.

Alkaline phosphatase : For alkaline phosphatase assay the 50 µl supernatant was added with 100 µl substrate solution (4 mg/ml p-nitrophenyl phosphate and 0.5 mM $MgCl_2$ in 1 M diethanolamine buffer, pH 9.8) the reaction proceeded at 37°C for 30 minutes. The absorbance was taken at 405 nm after stopping the reaction by addition of 100 µl 1 N NaOH.

β-**D-glucuronidase** : For β−D-glucoronidase assay, 100 µl supernatant was added with 100 µl of substrate solution containing 0.01 M p-nitrophenyl β−D-glucoronidase in 0.1 M sodium acetate buffer, pH 4. After incubation for 18 hours at 37^0C, the reaction was stopped by adding 100 µl of 0.4 M glycine and absorbance was measured at 400 nm.

Lactate dehydrogenase : This assay was performed based on the method of Anne Vassault (1983). The reaction mixture was composed of 80 mM Tris pH-7.2, 200 mM NaCl, 0.2 mM NADH, and 1.6 mM pyruvate and 50 µl homogenate in a total volume of 2.55ml reaction volume. The LDH activity was measured as by continuously monitoring the decrease in absorbance due to oxidation of NADH at 339nm for 3 min and the activity is expressed as nmol NADH oxidized/min per mg protein.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\textbf{LDH}} \text{L(+)-Lactate} + \text{NAD}^+$$

Measurement of GSH and GSSG:

U-937 cells (3 x 10^6/ml) were treated with 5 mM NAC and different concentrations of mangiferin. The cells were washed with PBS and lysed with 1% Triton X-100. GSH and GSSG were measured by enzymatic recycling assay described by Anderson. After 50 µl of 10% sulfosalisilic acid was added to each 100 µl aliquot of cell lysate, the samples were centrifuged (10,000x*g*) for 5 min at 4^0C to precipitate the protein. To assay total glutathione, a 50 µl aliquot of supernatant was added to a mixture of 0.7 ml of 0.3 mM NADPH, 100 µl of 6 mM DTNB, and 175 µl of H_2O. After adding 10 µl of 50 units/ml glutathione reductase, the rate of change in optical density at 405 nm was assessed. To assay GSSG, the GSH present in the sample was derivatized by adding 2 µl of 2- vinylpiridine and 6 µl of triethanolamine to a 100µl aliquot of supernatant. After 60 min of incubation at 25^0C, GSSG was measured in the same manner as GSH.

Measurement of catalase:

Cellular catalase values were determined using the method described in Beers and Sizer (1952). Briefly, U-937 cells were treated with 10 µg/ml of mangiferin and/or 5 mM of NAC for 6 h. Cells were pelleted, washed with PBS, lysed in 100 µl of 1% Triton X-100, and centrifuged at 10,000 rpm for 5 min. H_2O_2 was used as the substrate of catalase. Catalase was assayed by the disappearance of peroxide, which

was detected spectrophotometrically at 240 nm. The incubation mixture contained 50 mM potassium phosphate buffer, pH 7.4, 2 mM H_2O_2 and sample (enzyme source). The decrease in absorbance was recorded at 240 nm for 2 min.

Chemical crosslinking:

The level of receptor on the surface of neutrophils was detected through chemical crosslinking. The neutrophils ($1x10^7$ cells/tube) suspended in PBS were incubated with or without modulator(s) at 37°C. After incubation, the cells were washed, cooled and incubated with ^{125}I IL-8 (20 ng) at 37^0C. After binding, unbound ^{125}I IL-8 was removed by washing with D-PBS and finally the cells were suspended in 100 µl D-PBS. A bifunctional crosslinker DSS (1 mg/ml) was added to the cell suspension and incubated at 4°C for 1 hour. The cells were washed and mixed with 80 µl CHAPS (9 mM) in D-PBS with PMSF (200 µM), leupeptin (1 µM), pepstatin (1 µM) and EDTA (100 µM). After mixing thoroughly, centrifugation was carried out at 4°C at 10,000xg for 15 minutes. The supernatant was removed carefully, analyzed in 10% SDS-PAGE under reducing condition following the method of Laemmli. The gel was dried and autoradiograph was carried out using a Fuji Bas1000 phosphor imager.

Membrane Preparation:

Freshly prepared neutrophils ($1x10^8$/ml) were suspended in hypotonic buffer (10 nM HEPES containing 1 mM PMSF, 100 µM leupeptin, 100 µM pepstatin and 0.5 mM EDTA) were kept in ice for 30 minutes. Then the cells were homogenised in a Dounce homogeniser, centrifuged for 10 minutes at 160xg to remove unbroken cells and cell debris. The supernatant was layered on a discontinuous gradient of 5ml of 15% (w/v) sucrose resting on 10ml of 40% (w/v) sucrose and centrifuged at 1,00,000 x g for 60 minutes at 4°C. The pellet containing membrane was used for the experiment.

MTT assay:

The drug-induced cytotoxicity was measured by the MTT assay. Briefly 5000 cells per well (of 96 well plate) were incubated in the presence or absence of the indicated test sample in a final volume of 0.1 ml for 72 hours at 37^0C in triplicate assays. Thereafter 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After two hours of incubation at 37^0C, 0.1 ml of extraction buffer (20% SDS in 50% dimethlylformamide) was added. After an overnight incubation at 37^0C, the absorbance at 570 nm was measured using at 96 well multiscanner autoreader (Coda, Bio Rad) with the extraction buffer as blank.

Total RNA isolation from cultured mammalian cells:

To the cell pellet (approximately 2 million cells) 800 µl of TRIzol was added and the cells suspended in it by repeated pipetting. Later 160 µl of choloroform was added followed by vortexing for about 30 seconds. The tube was centrifuged at maximum speed for 5 minutes. The upper aqueous phase was transferred into a fresh microcentrifuge tube and 400 µl of ice-cold isopropanol added, this was incubated at -20°C for 1 hour. The RNA was pelleted by centrifugation at maximum speed for 15 minutes at room temperature. The supernatant was decanted and the pellet washed with 200 µl of 70% ethanol followed by centrifugation at maximum speed for 10 minutes. The supernatant was removed and the pellet dried under vacuum for about 5 minutes. The pellet was re-solubilized in 30-50 µl RNase free deionized (DEPC-treated) water and aliquots were stored at -70°C.

Detection of CXCR1 and CXCR2 by semiquantitative RT-PCR:

After treatment, total RNA was extracted using TRIzol (Gibco BRL) and 1 µg of total RNA was reverse-transcribed using poly-T oligonucleotide and M-MuLV reverse transcriptase (Invitrogen). The PCR was performed using primers for *CXCR1* (5'-ACACCCTCATGAGGACCCAG-3' and 5'-AGCATCCAGCCCTCATGAGG-3') and *CXCR2* (5'-CTATAGTGGCATCCTGCTAC-3' and 5'-CCAAGAAGAACCAGTGGACA-3'). Following PCR, the amplicons were analyzed by gel electrophoresis with ethidium bromide staining. The expression of

the investigated genes was determined by normalizing their expression against the expression of *actin* gene.

Western blot:

The proteins were resolved in a denaturing SDS-PAGE gel and after completion of the run the gel was over laid on a nitrocellulose paper cut to the size of gel and kept in the blotting cassette in the presence of blotting buffer. Finally the cassette was put in the mini transblot apparatus (Bio Rad) and blotting was done for 4 hours at a constant voltage of 60 V. Then the membrane was taken out and rinsed in PBS-T (Phosphate Buffer Saline – Tween 20) for 5 minutes by gentle shaking. Later the membrane was immersed in 5% non-fat milk solution in PBS-T with gentle shaking for 1 hour at 37°C. The membrane was washed off from the traces of the fat free milk with PBS-T and the membrane was over laid with primary antibody diluted in PBS-T for 3 hours at 4°C with shaking. After incubation the membrane was washed with PBS-T and layered with secondary antibody (conjugated with horse-radish peroxidase) diluted in 5% fat free milk solution (in PBS-T) and incubated for 45 minutes at room temperature. After incubation the membrane was washed and processed for the protein bands of interest using ECL-plus detection reagent (Amersham Biosciences) followed by development of the bands using X-ray film (Hyperfilm-ECL, Amersham Biosciences).

Preparation of silica slurry for DNA isolation:

50% (w/v) silica (powder silica SIGMA) was taken in sterile water and centrifuged to pellet. The supernatant was discarded and again loaded with water and the process was repeated 3 times. Finally fresh water was added to make 50% silica slurry. The slurry was aliquoted and used for DNA isolation.

Radioactive end labelling of oligos:

The oligonucleotides, ^{32}P-γATP, T4 Polynucleotide kinase buffer were thawed out. The following components were mixed in a microfuge tube on ice :-

Chemicals	Volume pipetted
oligo (1 p mole/µl)	3 µl
10X T_4 Polynucleotide kinase buffer	1 µl
Sterile H_2O	2.66 µl
^{32}P-γATP	3 µl
T_4 Polynucleotide kinase	0.33 µl
Total reaction volume	10 µl

This was mixed by tapping, pulse spin and incubated at 37ºC for 45 min so that enzymatic reaction occurs. The reaction was stopped by adding 2 µl of 0.5 M EDTA pH8.0. The column was prepared by inserting first glass wool in the 1 ml syringe and swollen Sephadex G-50 was added into the syringe. Now the column was taken and transferred to another improvised stand that contains a microfuge tube for the collection of elute. Then the reaction mixture was loaded on the top of the G-50 column. The eluting fractions were collected in the microfuge tube by loading 200 µl water on the top of the column. After collecting 5 to 6 fractions the tubes where analyzed for amount of radioactivity using a GM counter. The tube having between 7-10 x 10^6 counts was selected. That means the specific activity of the oligo would be between 3.5-4.5 x 10^6 cpm/pmoles. A labeling between 3.5-4.5 x 10^6 cpm/pmole would correspond to efficiency between 50 to 65 %. To the selected tube 100 pM of the complimentary strand of the oligo was added and the content boiled at 95°C for 5 minutes and allowed to anneal at room temperature for 3 to 4 hours.

Preparation of cytoplasmic and nuclear extracts:

To the fresh cell pellet in a micro centrifuge tube 200 µl of ice-cold cytoplasmic extract buffer was added and the pellet was suspended. The cell suspension was incubated on ice for 30 min. The buffer is hypotonic in nature, and cells are allowed to swell on ice. 6.2 µl of 10 % triton x100 (freshly prepared) for every 200 µl of cell

suspension was added to each microfuge tube. The cell suspension was vortexed vigorously for 15 seconds for the rupture of the plasma membrane. The contents were centrifuged for 1 min. at 10,000 rpm, supernatant (cytoplasmic extract) was then removed. If the cytoplasmic extract is to be saved then it is transferred to a pre-chilled microfuge tube and stored at –70ºC. 25 µl of ice-cold nuclear extract buffer was added to the pellet (nuclear pellet + membrane) in the microfuge tube. This was incubated on ice for 45 min with intermittent vortexing and then centrifuged for 5 min at 10,000xg. The supernatant (nuclear extract) was stored at –70ºC.

Protein estimation by Bradford method:

In the first well of a 96-well plate 50 µl of water was added in duplicate. To the subsequent wells 50 µl BSA standards (i.e. 50 µg/ml, 100 µg/ml) was added in duplicate. To the other wells diluted unknown protein sample (122.5µl of water + 2.5 µl of unknown protein extract) were added in duplicates. Then 200 µl of Bio-Rad reagent (1 ml reagent + 4 ml of and mixed) was added to each of the well including blank. The concentration of protein was read using the ELISA reader at 570 nm. The unknown protein concentration was calculated as follows:

$$X = \frac{\frac{\text{BSA Std 1(Conc)}}{\text{OD}} + \frac{\text{BSA Std 2 (Conc)}}{\text{OD}}}{2}$$

X x **50 (dilution factor)** / **1000** = **Y**

Concentration of unknown protein (µg/µl) = Y x its OD

Electrophoretic Mobility Shift Assay

The binding reaction-mixture was prepared. The constituents of which are as following:-

Stock Solutions	Volume pipetted
H_2O	6µl
10X Binding buffer	2 µl
Poly dI : dC (1 µg/µl)	2 µl
^{32}P-ds NF-κB oligo (4 fmoles/µl)	4 µl
10 % Triton x 100	2 µl
Total reaction volume	16 µl

4 µl of nuclear extract was added to the above mixture, mixed well and incubated at 37ºC in water bath for 1 hour. Tubes are transferred on ice; to it was added 4 µl of DNA-loading dye and mixed well by tapping. The sample was pulsed spinned to collect every thing to the bottom and the tubes were transferred on ice. The samples were loaded onto a native PAGE gel which was pre-run at constant voltage for 15 minutes. Electrophoresis was performed at 150 V (40 mA), till the bromophenol migrated 1-2 cm from bottom of the gel. A thin wedge-shaped article between the glass plates was inserted from one corner of the gel-mould and applied a twisting pressure to lift one of the glass plate (usually the top smaller one) carefully without disturbing the gel. A piece of Whatmann 3 paper was cut according to the size of the gel and was put on the gel. The paper was pressed gently and carefully and the gel was lifted, that is now firmly stuck on the paper. Then the gel was covered with saran wrap and kept on the gel-dryer at 80ºC for 1hr. under suction. After drying the gel, gel was removed from the gel-dryer, the corners were taped and was exposed on a Molecular-Dynamics Phosphorimager Screen to read the protein of interest.

Reporter Gene Assay:

Secretory alkaline phosphatase (SEAP):

Briefly, cells were transiently co-transfected by the lipofection method using 0.5 µg required plasmid DNA(s) with the protein of interest, a plasmid bearing NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (*SEAP*) gene

and β-*galactosidase* expression plasmid (Promega). By adding control plasmid *pCMVFLAG1* DNA, the total amount of DNA was maintained to 3 μg for each transfection. The cells were cultured for 12 hours and required stimulations done. Twelve hours later, cell culture-conditioned medium was harvested, and 25 μl of medium was mixed with 30 μl of 5xbuffer (0.5 M Tris, pH 9 and 0.5% bovine serum albumin) in a total volume of 100 μl in a 96-well plate and incubated at 65 °C for 30 min. The plate was chilled on ice for 2 min. Then 50 μl of 1 mM 4-methylumbelliferylphosphate was added to each well and incubated at 37 °C for 2 h. The activity of SEAP was assayed on a 96-well fluorescent plate reader (Fluoroscan II, Lab Systems, Needham Heights, MA) with excitation set at 360 nm and emission at 460 nm. The average number (+/- S.D.) of relative fluorescent light units for each transfection was then determined and reported as fold activation with respect to control vector transfected cells. β-Galactosidase activity was measured simultaneously using a β-galactosidase assay kit (Promega). The relative promoter activity was normalized with β-galactosidase activity as the transfection efficiency.

Luciferase (Luc) assay:

Briefly, cells were transiently co-transfected by the lipofection method using 0.5 μg required plasmid DNA(s) with the protein of interest, a plasmid bearing COX2 promoter DNA linked to Firefly Luciferase gene (Promega). The cells were cultured for 12 hours after transfection and required stimulations done. After 12h, cells were pelleted down and reporter gene was assayed as per the Promega protocol. Briefly, cells were lysed using the Lysis buffer provided with the kit. The samples were freeze-thawed twice by storing at -70^0C to ensure total lysis. The supernatant was obtained by centrifuging the same at 11,000 rpm for 2min. The supernatant was transferred to a fresh tube. 100 μL of the substrate was added to this and immediately read in the luminimeter.

Determination of lipid peroxidation:

Drug-induced lipid peroxidation was determined by detection of thiobarbituric acid (TBA)-reactive substances, which are the end products of polyunsaturated fatty acids

and related esters due to peroxidation. Cells (3×10^6/ml) pretreated without or with drug for required time were stimulated with stimulant for the required time. Then cells were washed with PBS and underwent three cycles of freeze thawing in 200 µl of water. A 20 µl of aliquot was removed, assayed for protein with Bradford protein determination and remaining samples were mixed with 800 µl of assay mix [0.4% (w/v) thiobarbituric acid, 0.5% (w/v) SDS, 9.4% (v/v) acetic acid, pH 3.5]. Samples were incubated for 60 min at 95 °C, cooled to room temperature, and centrifuged at 14,000 × *g* for 10 min, and the absorbance of the supernatants was read at 532 nm against a standard curve prepared using the MDA standard (10 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCl, pH 7.4). Results were calculated as nmol of TBARs equivalents/mg of protein expressed as a percentage of thiobarbituric acid-reactive substances above control values. Untreated cells showed 0.568 ± 0.08 nmol of TBA-reactive substances/mg protein (subtracting the background absorbance obtained by heating 800 µl of assay mix plus 200µl water).

Measurement of reactive oxygen intermediates:

The production of reactive intermediates mainly oxygen and nitrogen species upon treatment of cells with the inducer was determined by flow cytometry. Cells were exposed to dihydrorhodamine 123 (5 mM stock in DMSO) and then stimulated with drug at 37°C for 2 h, scrapped (in case of adherent cells) or pelleted (incase of suspension cells), washed and resuspended in 1 ml D-PBS at 0.5×10^6 concentration. Rhodamine123 fluorescence intensity resulting from dihydrorhodamine 123 oxidation was measured by a FACScan flow cytometer with excitation at 488 nm and was detected between 515 and 550 nm. Data analysis was performed using LYSYSII software (Becton Dickinson).

^{3}H-Thymidine incorporation assay:

The viable and proliferating cell number was detected by ^{3}H-Thymidine incorporation assay. U-937 cells (10^4 cells/well of 96-well plate) were incubated with test sample in a final volume of 0.2 ml for 72 h at 37°C. Cell proliferation was measured by thymidine incorporation by adding 50 µl of ^{3}[H]thymidine (0.5

μCi/well diluted in Hank's buffered salt solution) lasting for 18 h. Cells were harvested and washed, and thymidine incorporation was measured in a beta counter (Packard).

Immunocytochemistry:

The level of desired protein was examined by the immunocytochemical method as described with slight modifications. Briefly, cells were plated on a poly-L-lysine-coated glass slide air-dried, fixed with 3% formaldehyde, and permeabilized with 0.1% of Triton X-100. Blocked with 5% serum for 1h, washed with PBS-T and slides were incubated with anti-primary antibody Ab for 6 h followed by incubation with anti-rabbit IgG (secondary)-Alexa Fluor for 1 h. Slides were mounted with mounting medium with DAPI and analyzed under a fluorescence microscope.

Live/Dead assay:

The cytotoxic effects were also determined by the Live/Dead assay (Molecular Probes, Eugene, OR). Briefly, after different treatment 1 x 10^5 cells were stained with Live/Dead reagent (5 μM ethidium homodimer, 5 μM calcein-AM) and then incubated at 37^{0}C for 30min. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan).

Ultra competent cells preparation:

All the salts (10 mM PIPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 55 mM $MnCl_2$. $2H_2O$) except $MnCl_2$ were dissolved in water and pH was adjusted to 6.7 with 1N KOH. $MnCl_2$ was dissolved separately in water. $MnCl_2$ was added drop wise while stirring ($MnCl_2$ if added directly will give a brown color to the solution and precipitate out, hence it needs to be dissolved separately). Solution was then filter sterilized and stored. To prepare competent cells pre-inoculum was prepared. A single bacterial colony was picked from LB agar plate that has been incubated for 16-20 hours at 37°C and inoculated into 3 ml LB medium and incubated overnight at 37°C temperature with 200 rpm shaking. 1% of this pre-inoculum was subcultured in 100 ml LB-broth and incubated at 18°C until OD_{600} reached 0.5 - 0.6 (approx.).

Culture was kept on ice for 10 min. with constant shaking. Cells were pelleted by centrifugation at 2000xg/4°C/8 min. Pellet was resuspended in 40 ml of ice-cold TB buffer. Bacterial suspension was kept on ice for 30 min, re-spun at 200xg/4°C /8 min. Pellet was resuspended in 8 ml of TB buffer in which final concentration of DMSO was 7% and left on ice for 10 min.100 µl aliquots were made and snap freezed in liquid nitrogen and stored at -80°C.

Preparation of plasmid DNA by alkaline lysis:

Grown culture was pelleted by centrifugation at 10,000xg/4°C/3 min supernatant was discarded. Pellet was resuspended in 250 µl of ice-cold alkaline lysis solution - 1. 300 µl of alkaline solution - 2 was then added and the tuber was inverted very gently 3-4 times and incubated at room temperature for 5 min. 350 µl solution - 3 was added and mixed by inverting the tube 3 or 5 times, gently. Suspension was incubated on ice for 10 min. Bacterial lysate was spun at 10,000xg/12 min/4°C. Supernatant was transferred to a fresh tube. 0.4 volume of phenol:chloroform was added to the supernatant. Inverting the tubes several times mixed contents. It was then spun at 10,000xg/12 min/4°C. Aqueous phase was taken out in a fresh tube and 0.6 volume of isopropanol was added, mixed properly and incubated at room temperature for half an hour and spun at 10,000xg/RT/20 min. Supernatant was discarded. Pellet was washed with 70% ethanol. The tube was stored at room temperature until the ethanol has dried. The pellet was resuspended in 20 µl of water and 20 µg/ml RNase added. The tube was incubated at 50°C for 45 min. the tube was vortexed for few seconds. Concentrations of the vectors were then estimated by running 1% agarose gel.

Spectrophotometric estimation of nucleic acids

The quantity and purity of nucleic acids was determined by measuring the absorbance at 260 and 280 nm. The concentration of nucleic acids was calculated by taking 1 OD_{260}= 50 µg/ml for DNA, 40 µg/ml for RNA and 33 µg/ml for single stranded oligonucleotides. The purity of nucleic acids was checked by their A_{260}/A_{280} ratio.

Construction of GST-c-Jun.

PCR cloning:

PCR amplification of the N-terminal domain encoding the phosphorylation region of c-Jun from peripheral blood.

Primer sequence

1) c-JunBam+ 5'-ATC GG**G GAT CC**G ATG ACT GCA AAG ATG GAA-3'

2) c-JunXho- 5'-CTA GGG **CTC GAG** TCA GGG GCA CAG GAA CTG GGT-3'

Reaction mixture (25 μl):-

Template	1.0 μl
Primer 1	0.5 μl
Primer 2	0.5 μl
dNTPs (2.5mM)	1.0 μl
10X buffer	2.5 μl
Enzyme Deepvent	0.5 μl
Water	19.0 μl
	25 μl

PCR parameters:

1) Pre-denaturation	94°C, 2 min
2) Denaturation	94°C, 30 secs
3) Primer annealing	54°C, 1 min
4) Elongation	72°C, 45 secs
5) Final elongation	72°C, 7 min

The PCR amplified product was purified through agarose gel. c-Jun was ligated to pET41b (linearised prior by BamHI and Xho-) vector, using T4 DNA ligase at 16°C for 12 hours. The constructs so obtained were then transformed into DH5α competent cells and the positive colonies were selected. Clones were confirmed by BamHI.

Protein extraction and purification using Glutathione Sepharose 4B:

A primary inoculum of 3 ml of LB broth containing 30 µg/ml kanamycin was prepared by inoculating it with one colony of E. coli BL21 (DE3) harboring pET41b construct and grown O/N at 37°C. 100 ml of LB broth with kanamycin was inoculated with overnight grown preinoculum and incubated at 37°C with vigorous shaking until OD reached 0.5. At this stage it was induced with 1mM IPTG and grown further for 4 hours at 37°C.The culture was spun at 1000 rpm for 15min at 4°C. Supernatant was discarded and the pellet resuspended in lysis buffer (pH 8) (50 mM NaH_2PO_4 and 300 mM NaCl) and 1 mM PMSF added and was sonicated for 10 min at 20% power output and 30 sec on -off cycle. After sonication, lysate was spun at 500xg/30 min/4°C. The overexpressed protein was purified using Glutathione Sepharose 4B matrix column. The column was packed with Glutathione Sepharose 4B along with 500 µl of 10%Triton x 100 and the supernatant collected after the lysis, sonication and spinning of 100 ml culture (induced with IPTG), incubated at RT with gentle agitation for 40 min. Spun at 700 rpm for 5min. Pellet was washed with 10 bed volumes of PBS. After repeated wash at100xg to the sedimented matrix, 1.0 ml of elution buffer per ml bed volume of GSH Sepharose 4B was added. Resuspended gently, incubated at room temperature for 10 minutes, and elutes were collected and saved for SDS-PAGE analysis.

Kinase Assay:

After treatment of cells ($3x10^6$ /ml) with the drug for required time interval, cell extracts were prepared by lysing cells in buffer containig 20 mM HEPES, (pH 7.4), 2 mM EDTA , 250 mM NaCl, 1% Nonidet- P-40, 2 µg/ml leupeptin , 2 µg/ml aprotinin, 1 mM PMSF, 0.5 µg/ml benzamidine and 1 mM DTT. Cell extracts (250 µg/sample) were immunoprecipitated with 0.3 µg of anti-JNK antibody or anti-ERK for 3 hours at 4°C. Immunocomplexes were collected by incubation with protein A/G-Sepharose beads for 1 hour at 4°C the beads were extensively washed with lysis buffer (4 x 400 µl) and kinase buffer (2 x 400 µl, 20 mM HEPES (pH 7.4), 1 mM DTT, 25 mM NaCl). Kinase assays were performed for different time intervals at 30°C with GST-c-Jun or matrix protein as substrates in 20 mM HEPES, (pH7.4), 10 mM $MgCl_2$, 1 mM DTT, and 10 µCi of ^{32}P- γ ATP. Reactions were stopped with the addition of 20 µl of 2x SDS sample buffer, boiled for 5 min and subjected to SDS-PAGE (10%). GST-Jun was visualized by staining with Coomassie brilliant blue and the dried gel was analyzed by a Phosphor Imager.

CHAPTER THREE

ACTION OF α-MELANOCYTE STIMULATING HORMONE IN MAST CELLS

3.1 Introduction

Research over the last two decades has revealed a close link between the Neuroendocrine and the Immune system. α-Melanocyte Stimulating Hormone (α-MSH) is a classic example of a mediator establishing in such a link. α-MSH (SYSMEHFRWGKPV) is a tridecapeptide, formed by the post translational enzymatic cleavage of Pro-opimelanocortin hormone (POMC). Although the receptors for α-MSH were primarily identified in the cells of the nervous system and the melanocytes, it has been shown to be expressed by a variety of other extra pituitary cells including monocytes, mast cells, macrophages, and neutrophils all of which are involved in the immune regulation (Star *et al.,* 1995; Catania *et al.,* 1996). Thus becomes intriguing to understand the significance of the presence of α-MSH receptors on various cells of the body and to study how it modulates the functions of all these cells.

Mast cells play a central role in inflammatory and immediate allergic reactions. These cells release preformed mediators, newly synthesized mediators, as well as cytokines upon antigen activation thereby forming the genesis of inflammation (Galli *et al.,* 1991). Hence, the regulation of mast cell activity becomes important. Mast cells reside immediately beneath the thin epithelial lining of respiratory duct and when exposed to allergens produce multi-mediators for allergic responses. Mast cells are also found in the human heart (Dvorak AM, 1986), and have been implicated in cardiovascular diseases (Marone *et al.,* 1995; Panizo *et al.,* 1995) as mast cell-derived mediators cause apoptosis of cardiac myocytes and proliferation of non-myocytes (Hara *et al.,* 1995) leading to hypertrophied and failing hearts (Patella *et al.,* 1998).

Among the several signaling pathways of the immune system activated by any injurious signal, NF-κB is an important one. As NF-κB is involved both in cell proliferation and inflammation, the regulation of this transcription factor will be helpful to regulate the mast cell number, thereby mast cell-mediated inflammation. MSH treatment has been shown to inhibit the activation of NF-κB by TNF-α or LPS in monocytes and this inhibition is demonstrated to be dependent on cAMP (Manna and

Aggarwal, 1999). This inhibition is not cell type specific. However, detailed molecular mechanism of action of MSH is still not clearly understood. Since we detected receptors for α-MSH on mast cells, we were interested to understand the molecular mechanism of modulation of mast cells by MSH. To mimic an inflammatory situation, we used for our study LPS-LBP complex named as SA-LPS (serum activated LPS).

3.2 Results

In this study, we examined the effect of α-MSH on the regulation of mast cell activity. We used murine mast cell line (MC-9) for the work because these cells express sufficient α-MSH receptors.

α-MSH inhibits SA-LPS-induced NF-κB activation.

To detect the role of α-MSH on endotoxin-induced NF-κB activation in murine mast cells (MC-9), cells were stimulated with SA-LPS (100 ng of LPS was incubated with 20 μl of serum for 1 h at 37^0C and this mixture was SA-LPS) for 2h at 37^0C and then treated with different concentrations of α-MSH for 24 h at 37^0C. Nuclear extract (NE) was prepared and 8 μg NE proteins were analyzed in 6.6% native PAGE to detect NF-κB by gel shift assay. The results indicated in Figure 3.1.1 showed that α-MSH alone did not activate NF-κB, but it inhibited SA-LPS-induced NF-κB activation in a dose dependent manner. At 10^4 pM concentration, α-MSH completely abrogated SA-LPS-induced NF-κB activation. Various combinations of Rel /NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. To show that the retarded band visualized by EMSA in SA-LPS-induced cells was indeed NF-κB, we incubated the nuclear extracts from SA-LPS-activated cells with antibodies (Abs) against p50 (NF-κBI) and p65 (Rel A) or in combination and then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher m.w. (Fig.3.1.2), thus suggesting that the SA-LPS-activated complex consisted of p50 and p65 subunits. MC-9 cells were exposed to SA-LPS for 1 h and then treated with α-MSH for 0 to 36 h and then NF-κB was assayed from

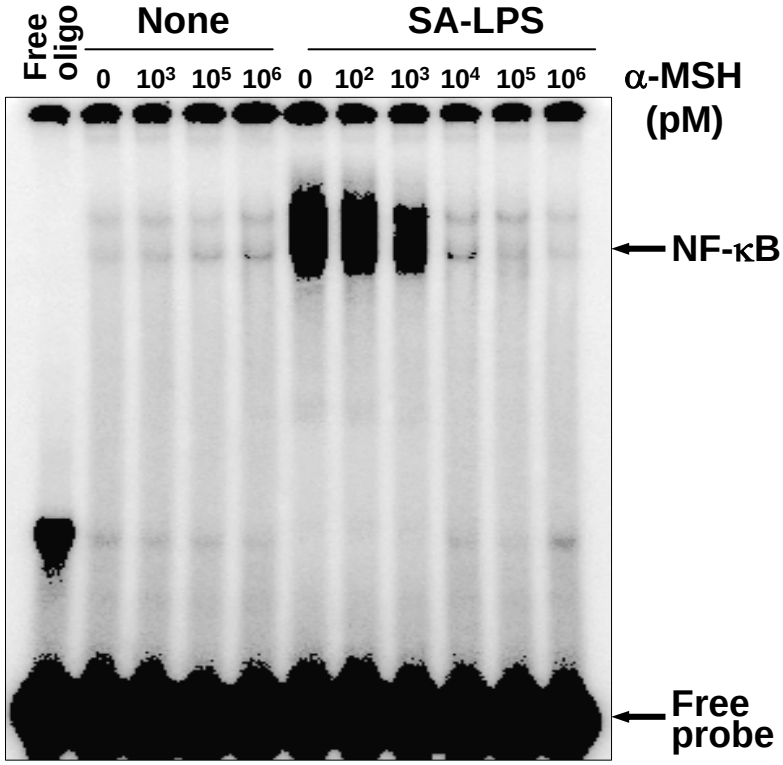


Fig.3.1.1 Effect of α-MSH on SA-LPS-induced NF-κB activation. MC-9 cells (2x 10^6/ml) were stimulated with SA-LPS (100 ng/ml) for 2 h and then treated with different concentrations of α-MSH for 24 h at 37^0C, CO_2 incubator. NF-κB was assayed from 8 μg NE proteins

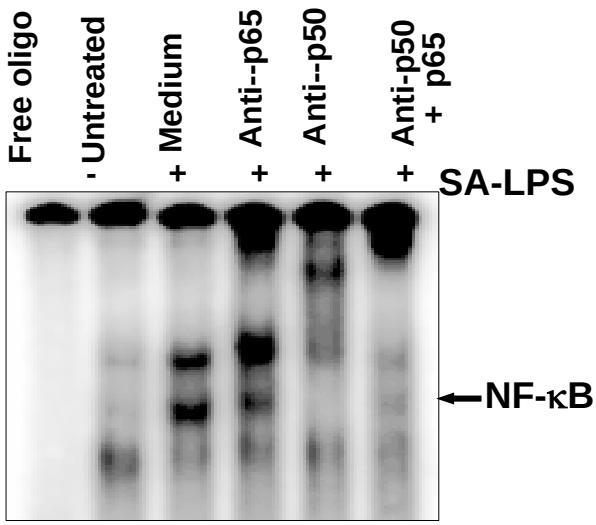


Fig. 3.1.2 Supershift of NF-κB band. SA-LPS -induced NE was incubated for 15 min with different Abs and then assayed for NF-κB.

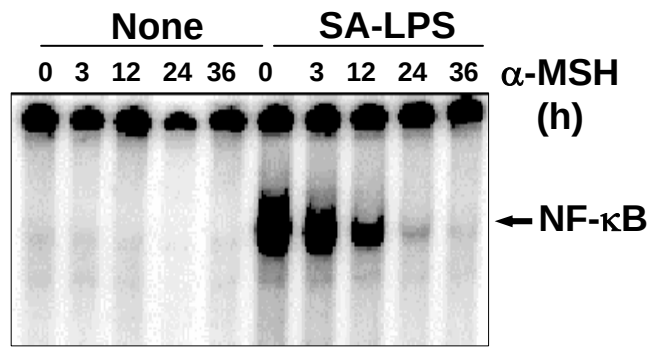


Fig 3.1.3 Optimum time of α-MSH treatment to downregulate SA-LPS-induced NF-κB activation. MC-9 cells, pretreated with or without SA-LPS were treated with α-MSH (10 nM) for 0 -36 h and then NF-κB was assayed.

nuclear extract. SA-LPS-induced NF-κB activation was inhibited maximally when the cells were treated for 24 h with α-MSH (Fig.3.1.3). Overall, these results indicate that α-MSH inhibits SA-LPS induced NF-κB.

α-MSH inhibits SA-LPS-induced NF-κB reporter gene activation.

As SA-LPS-induced NF-κB activation was blocked by α-MSH, the NF-κB dependent gene expression was also carried out. MC-9 cells were transfected with NF-κB reporter plasmid containing *SEAP* gene and/or *IκBα-DN* plasmid. Cells were stimulated with SA-LPS for 4 h and replaced with fresh medium. Cells were then treated with α-MSH for 24 h. Culture supernatant was collected and used to assay secretory alkaline phosphatase activity. The result in Figure 3.1.4 indicated that SEAP activity was induced with increased concentrations of SA-LPS. α-MSH (10 nM) completely inhibited SEAP activity at any concentrations of SA-LPS. The IκBα-DN transfected cells showed the basal activity of SEAP when stimulated with SA-LPS indicating the specificity of the assay.

α-MSH inhibits SA-LPS-induced ICAM-1 expression.

As α-MSH inhibited SA-LPS-induced NF-κB and its dependent reporter gene, we looked for the status of adhesion molecule ICAM1, a NF-κB-dependent gene product. Cells were stimulated with different concentrations of SA-LPS for 2 h and then treated with α-MSH (10 nM) for 24 h. Then 100 μg of whole cell extract proteins were analyzed using 9% SDS-PAGE and ICAM1 was detected by Western blot analysis. SA-LPS induced ICAM-1 expression in a dose-dependent manner, which was inhibited by α-MSH (Fig.3.1.5).

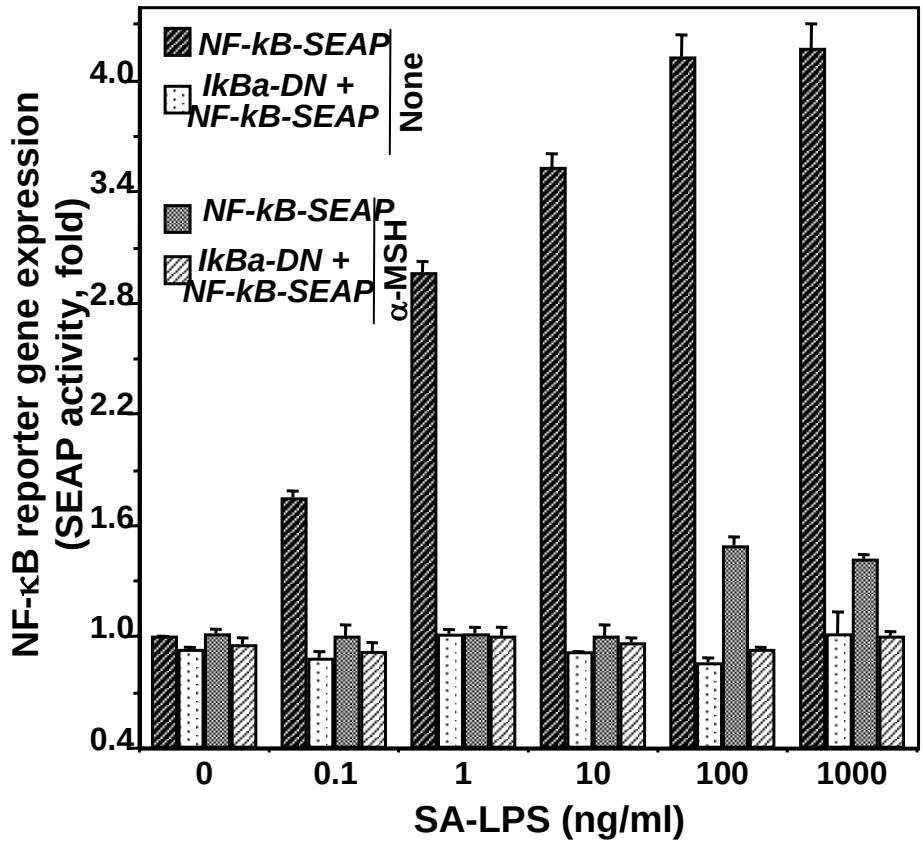


Fig. 3.1.4 Effect of α-MSH on SA-LPS-induced NF-κB- dependent reporter gene expression. MC-9 cells were transiently transfected with indicated plasmids along with NF-κB responsive promoter plasmid linked to the SEAP gene. The cells were cultured for 12 h and then stimulated with different concentrations of SA-LPS for 4 h as shown in the figure. Cells were then treated with 10 nM α-MSH for another 24 h. Culture supernatant was taken and assayed for SEAP.

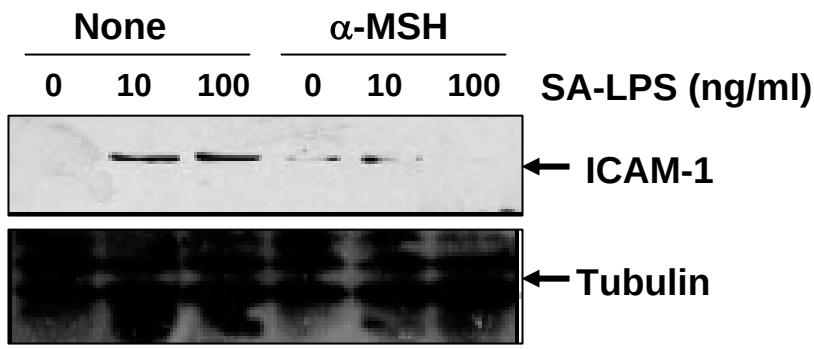


Fig. 3.1.5 Effect of α-MSH on SA-LPS-induced ICAM-1 induction. Cells were stimulated with different concentrations of SA-LPS for 2 h and then treated with α–MSH (10 nM) for 24 h at 37^0C, CO_2 incubator. ICAM-1 was detected from cell extract proteins (100 μg) by Western blot analysis. Tubulin was detected by reprobing the same blot.

α-MSH receptors in different cells.

To understand the specificity of α-MSH-mediated downregulation of NF-κB, we detected the level of expression of α-MSH receptors in different cells. H4, HeLa, and Raw 264.7 cells (1 x 10^6/well) were plated and incubated for overnight in 12-well plate. MC-9, U-937, and Jurkat cells (1 x 10^6/sample) were kept on ice in triplicate. Cells were incubated with 4 ng ^{125}I-labeled α-MSH (5 x 10^4 cpm) for 2 h at 4^0C in presence or absence of 50 fold cold α-MSH and α-MSH binding was assayed. The results represented as mean specific binding in cpm ± SD of triplicate samples (Fig.3.2.1). The results indicate that the α-MSH receptors are expressed in MC-9, Jurkat, and Raw 264.7 cells, which correlate with α-MSH mediated downregulation of SA-LPS-induced NF-κB activation. α-MSH binds with its cell surface receptor specifically melanocortin-1 receptor (MC-1R). The levels of MC-1R were detected using 200 μg of different cells extract proteins by Western blot analysis (Fig.3.2.2).

Inhibition of NF-κB activation by α-MSH is cell type specific.

As NF-κB activation pathways differ in many cell types (Baeuerle and Baichwal, 1997; Bonizzi *et al.,* 1997; Li N and Karin M, 1998; Imbert *et al.,* 1996), we therefore studied whether α-MSH affects human Jurkat (T) cells and H4 (glioma) cells and murine macrophage (Raw 264.7) cells as well. It has been demonstrated that distinct signal transduction pathways could mediate induction in epithelial, neuronal, and lymphoid cells. All the effects of α-MSH described above were conducted with MC-9 cells we also tested its effect on other cell types. We found that α-MSH blocks SA-LPS-induced NF-κB activation in Jurkat cells completely, Raw 264.7 cells partially, but not in H4 cells (Fig.3.2.3) suggest that this effect of α-MSH is extended to T-cells but not glioma cells.

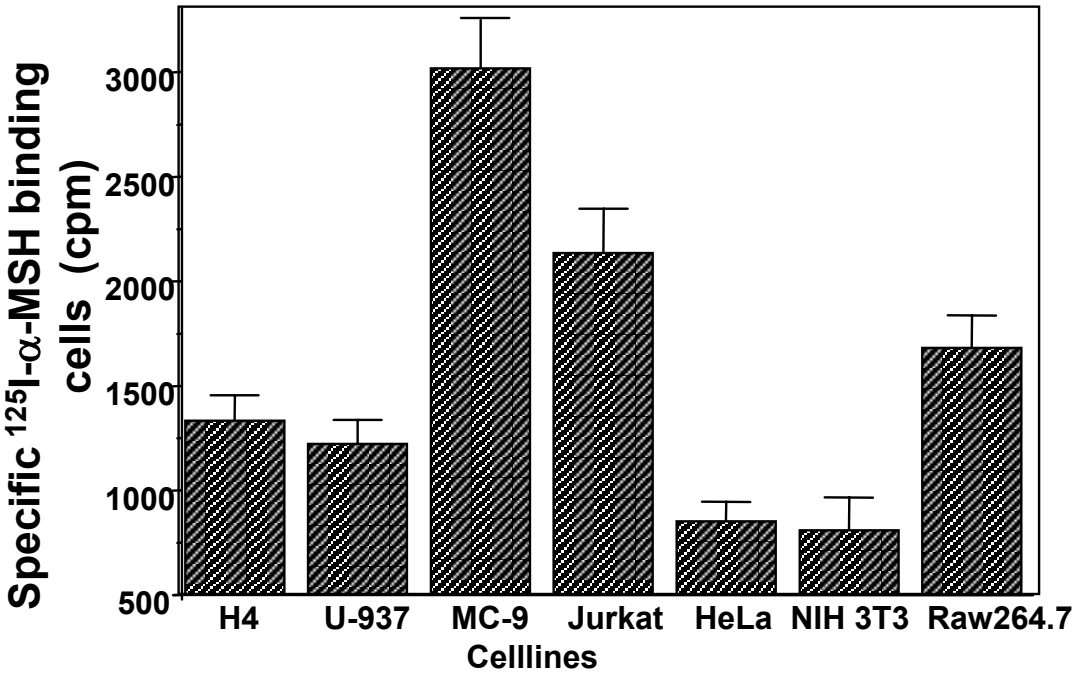


Fig 3.2.1 Levels of α-MSH receptors in different cells. U-937, MC-9, and Jurkat ($1x10^6$) cells were taken in tubes as triplicate sets.H4, HeLa, NIH 3T3, and Raw 264.7 cells were cultured in a 12-well plate as triplicate at 37^0C CO2 incubator. Cells were incubated with 4 ng iodinated α-MSH ($5x10^4$ cpm) per sample in presence or absence of 200 ng of unlabeled α-MSH for 2 h at 4^0C. Then labeled α-MSH binding was assayed as described in Materials and Methods.

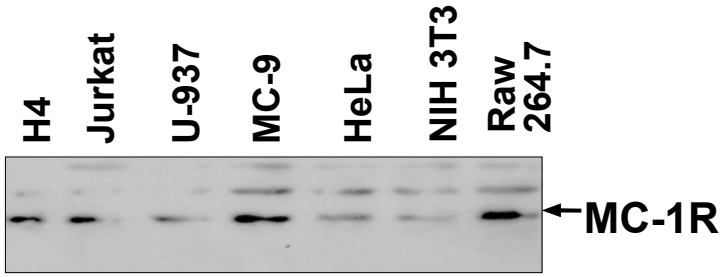


Fig.3.2.2 Level of MC-1R in different cell lines. Different cells' extract (200 μg) was analyzed in 9% SDS-PAGE and detected for MC-1R using anti-MC-1R antibody.

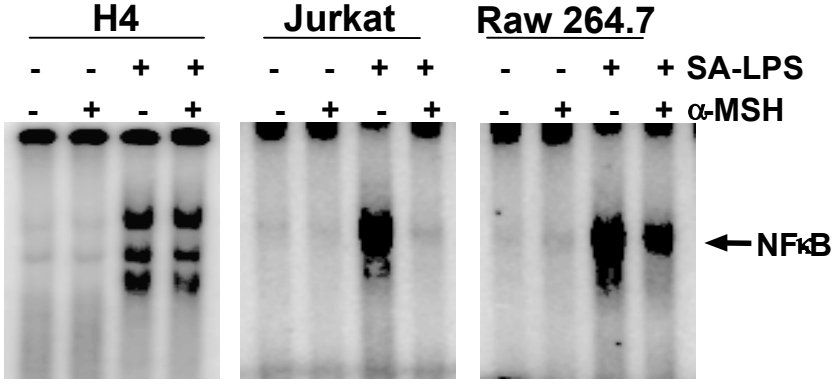


Fig.3.2.3 Effect of α-MSH on SA-LPS-induced NF-κB activation in different cell lines. Human glioma (H4), T-cell (Jurkat), and mouse macrophage (Raw 264.7) cells were stimulated with SA-LPS for 2 h and then incubated with 10 nM α-MSH for 24 h, 37^0C. After these treatments, NEs were prepared and then assayed for NF-κB.

α-MSH induces cell death in MC-9 cells.

To investigate the effects of α-MSH on induction of cell death in MC-9 cells different parameters (cell viability, thymidine incorporation, lipid peroxidation, reactive oxygen intermediates generation, caspase 8, and PARP cleavage) were assayed.

α-MSH induces cell death and inhibits ^{3}H-thymidine incorporation.

To detect α-MSH mediated cell viability, MC-9 cells were stimulated with SA-LPS (100 ng/ml) for 2 h and then treated with different concentrations of α-MSH for 36 h as indicated. Then cell viability was assayed by MTT assay and cell proliferation was assayed by ^{3}H-Thymidine incorporation by MC-9 cells. As shown in Figure 3.3.1, α-MSH induced cell death in a dose dependent manner in MC-9 cells. SA-LPS stimulated cells showed about 20% increase in cell viability than un-stimulated cells. SA-LPS was unable to protect α-MSH-mediated induction of cell death. Almost similar results were obtained with ^{3}H-thymidine incorporation by α-MSH treated cells (Fig.3.3.2). The results indicate that α-MSH alone causes cell death in MC-9 cells and SA-LPS does not protect from α-MSH-mediated cell death.

α-MSH induces lipid peroxidation in MC-9 cells.

As lipid peroxidation is a marker of cell death, we examined the effect of α-MSH on lipid peroxidation in MC-9 cells through the detection of levels of malondialdehyde (MDA) production. As shown in cell viability, α-MSH induced lipid peroxidation in a dose dependent manner and SA-LPS did not protect α-MSH-mediated lipid peroxidation (Fig.3.3.3 A).

α-MSH induces ROI generation in MC-9 cells.

ROI generation is an intermediate step in cell death induced by different agents. To detect the role of α-MSH on SA-LPS-induced ROI generation, MC-9 cells were stimulated with 100ng/ml of SA-LPS for 2 h and then treated with different

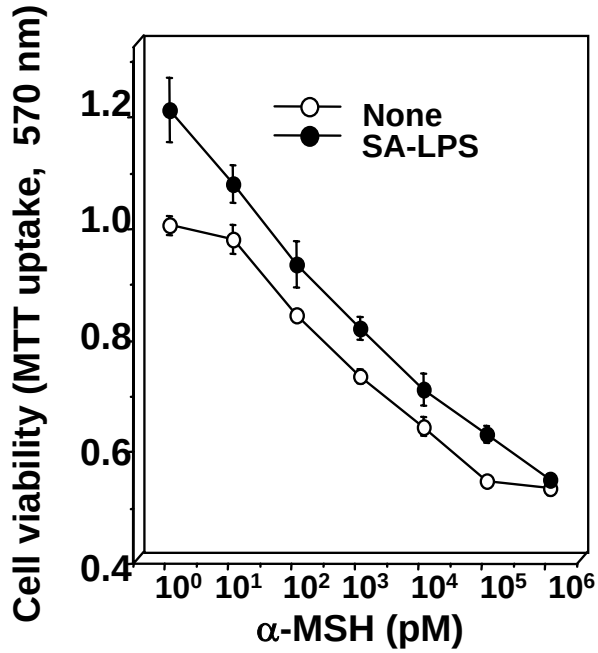


Fig. 3.3.1. Effect of α-MSH on cell viability in SA-LPS-induced MC-9 cells. MC-9 cells were stimulated with 100 ng/ml SA-LPS for 2 h and then 10^4 cells were taken in per well of a 96-well plate. Cells were then treated with different concentrations of α-MSH for 36 h and cell viability was assayed by MTT uptake.

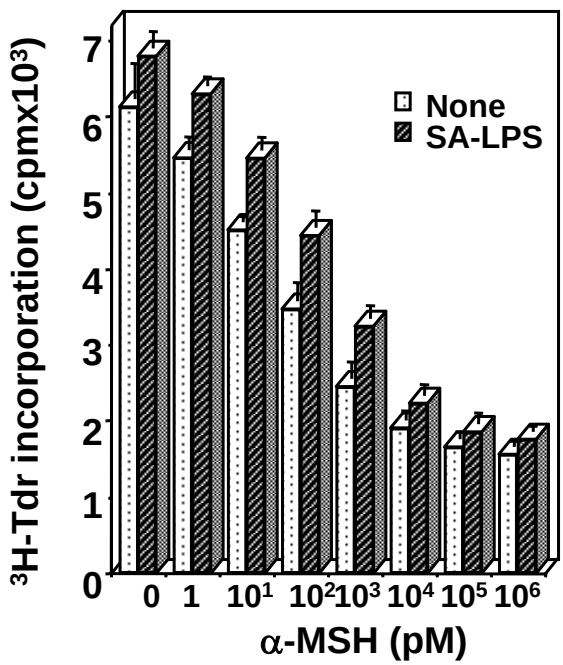


Fig. 3.3.2. Effect of α-MSH on cell viability in SA-LPS-induced MC-9 cells. MC-9 cells were stimulated with 100 ng/ml SA-LPS for 2 h and then 10^4 cells were taken in per well of a 96-well plate. Cells were incubated with 0.5 μCi of ^{3}H-thymidine (Tdr) for last 18 h and then assayed for Tdr incorporation in the cells as described in Materials and Methods.

concentrations of α-MSH for 36 h. Then ROI generation was examined with dihydrorhodamine dye conversion to rhodamine as described in Materials and methods. α-MSH-mediated ROI generation was shown in a dose dependent manner. SA-LPS-induced ROI generation was further increased with increased concentrations of α-MSH indicating its additive effect for ROI generation (Fig.3.3.3 B).

α-MSH induces Caspase 8 and PARP cleavage in MC-9 cells

Cell death is reflected with activation of caspases, which cleave a lot of proteins including PARP. Caspase 8 activation was shown by its auto-cleavage of precursor caspase8 into cleaved p20 caspase8. α-MSH (10 nM) not only induced the cleaved p20 caspase8 alone but also SA-LPS pre-stimulated cells as well (Fig.3.3.4). The level of precursor caspase8 was increased by α-MSH treatment. The same blot was reprobed for tubulin protein and the intensity of bands in all these lanes were equal suggesting loading control. α-MSH (10 nM) or TNF (1 nM) at 36h induced PARP cleavage individually as detected by Western blot (Fig.3.3.5). In SA-LPS pre stimulated MC-9 cells, α-MSH induced PARP cleavage indicating that SA-LPS were unable to protect α-MSH-mediated cell death.

α-MSH inhibited SA-LPS-induced NF-κB activation, induced cell death and PARP cleavage in human mast cells and mouse bone marrow-derived mast cells

As shown α-MSH-mediated inhibition of endotoxin-induced NF-κB activation in murine mast cells (MC-9), this effect was also tested in human mast cell line, HMC-1 and mouse bone marrow-derived mast (BM-mast) cells. Cells, stimulated with autologous serum activated LPS (100 ng/ml) for 2h at 37^0C were treated with different concentrations of α-MSH for 24 h at 37^0C and NF-κB was assayed from nuclear extracts. The results indicated that α-MSH alone did not activate NF-κB, but SA-LPS-induced NF-κB activation was inhibited in a dose dependent manner both in HMC-1 (Fig.3.4.1A) and BM-mast (Fig.3.4.1B) cells. α-MSH (10 nM) induced PARP cleavage in time

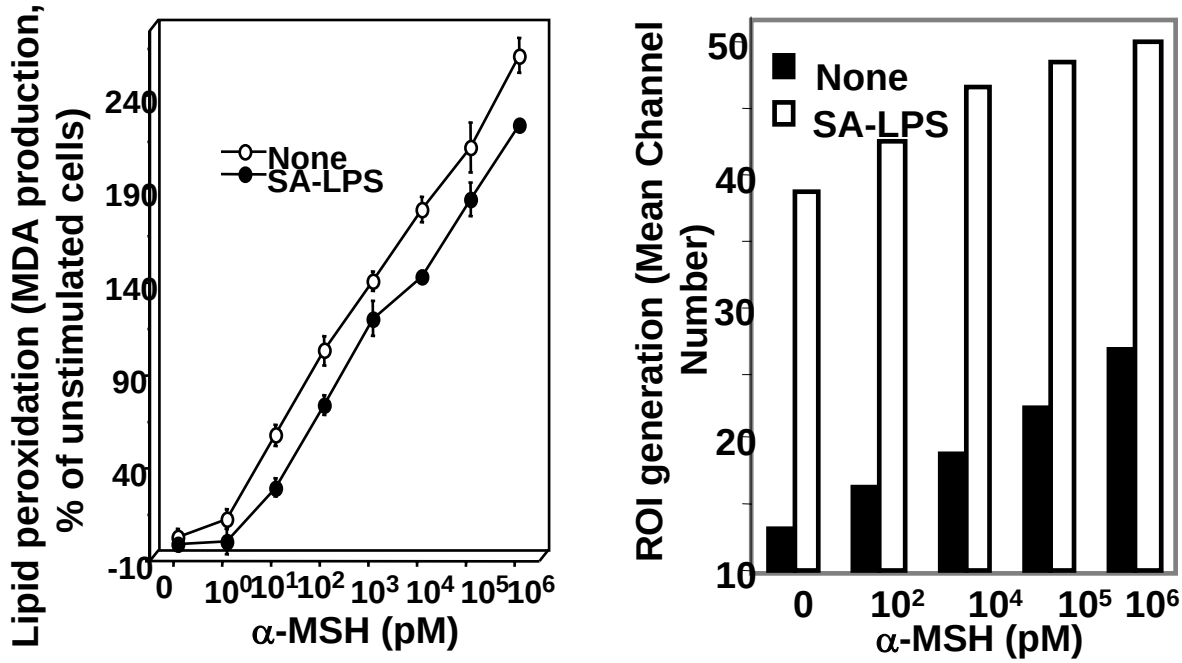


Fig. 3.3.3. A. Effect of α-MSH on lipid peroxidation SA-LPS-induced MC-9 cells. Cells were stimulated with different concentrations of α-MSH for 24h. Lipid peroxidation was assayed by measuring MDA as represented in percentage above unstimulated cells. **B. Effect of α-MSH on ROI generation in SA-LPS-induced MC-9 cells.** Cell were stimulated with SA-LPS for 2 h and then treated with different concentrations of α-MSH for 24 h. ROI generation was assayed as the mean channel number by dihydrorhodaminein flow cytometer.

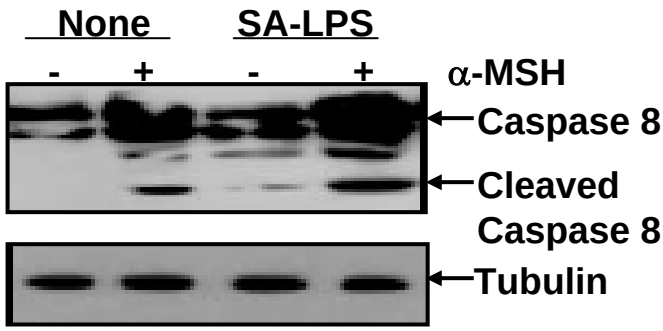


Fig.3.3.4. Effect of α-MSH on caspase8 in SA-LPS-stimulated cells. MC-9 cells were stimulated with SA-LPS for 2 h and then treated with α-MSH(10 nM) or TNF (1 nM) for 36 h. Then cell extract proteins were assayed for caspase8 by Western blot.

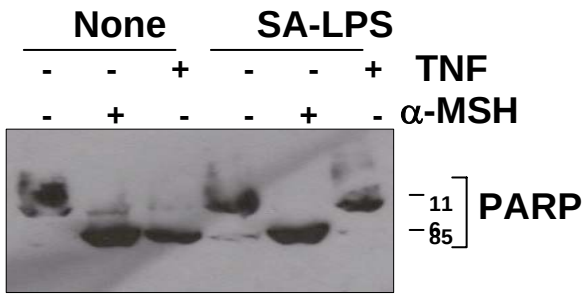


Fig.3.3.5. Effect of α-MSH on PARP cleavage in SA-LPS-stimulated cells. MC-9 cells were stimulated with SA-LPS for 2 h and then treated with α-MSH (10 nM) or TNF (1 nM) for 36 h. Then cell extract proteins were assayed for PARP by Western blot.

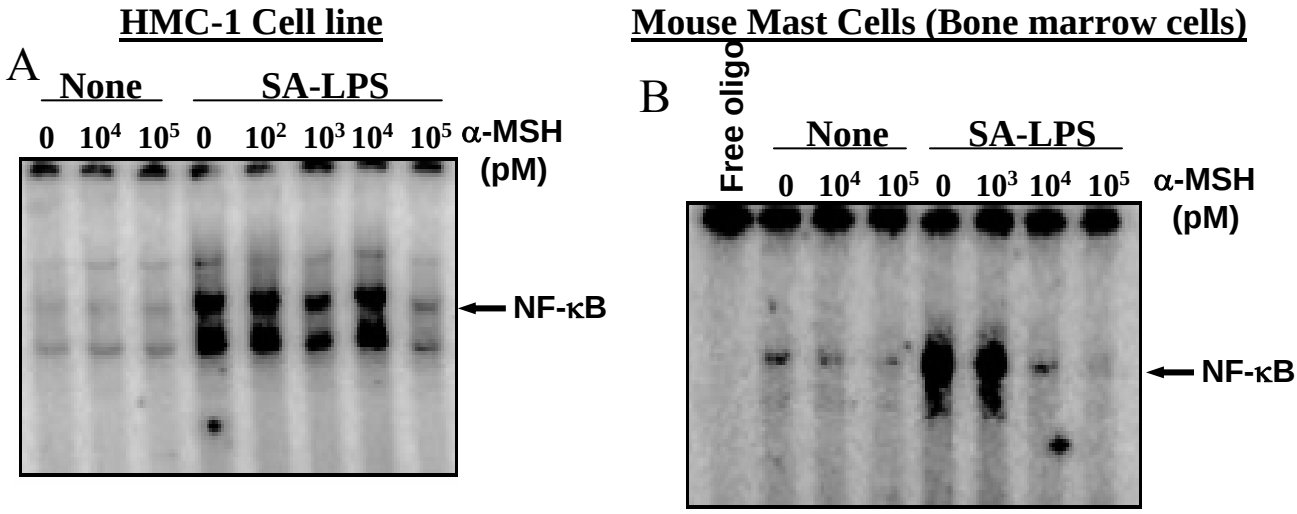


Fig. 3.4.1 Effect of α-MSH on SA-LPS-induced NF-κB activation in HMC-1 and bone marrow-derived primary mast cells. HMC-1 (**A**) and BM-mast cells (**B**) were stimulated with SA-LPS (100 ng/ml) for 2 h and then treated with different concentrations of α-MSH for 24 h at 37^0C, CO_2 incubator. NF-κB was assayed from 8 μg NE proteins.

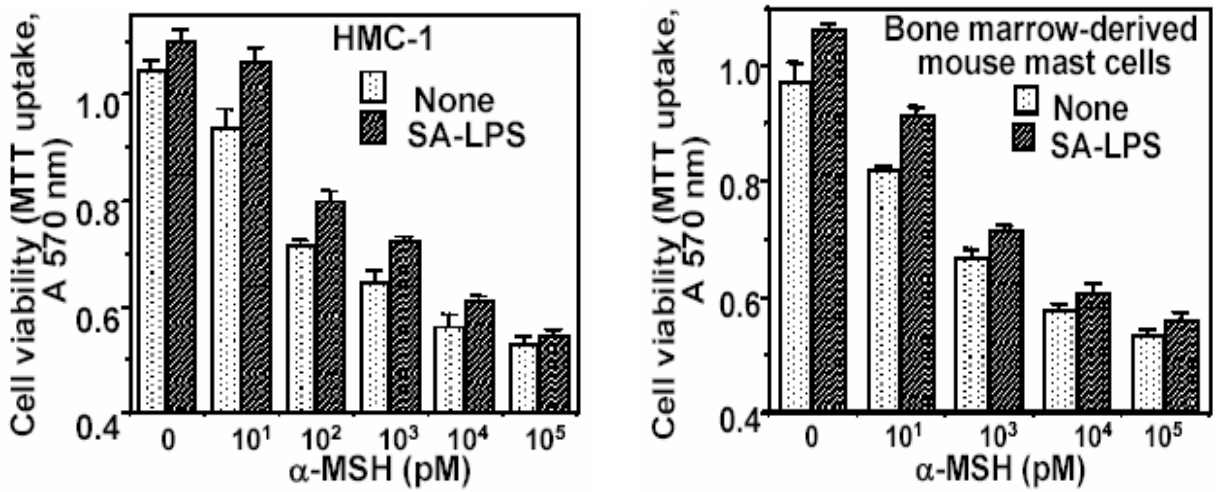


Fig.3.4.2. Effect of α-MSH on cell viability in SA-LPS-induced HMC-1 and BM-mast cells. Cells (10^4/well) were stimulated with 100 ng/ml SA-LPS for 2 h and then treated with different concentrations of α-MSH for 36 h and cell viability was assayed by MTT uptake.

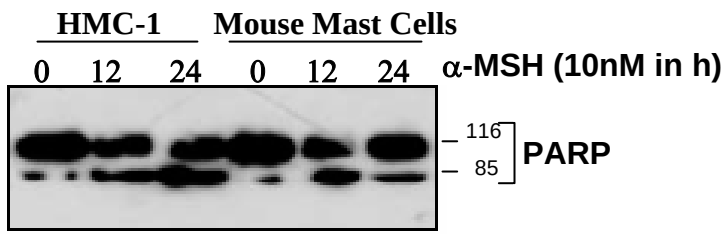


Fig. 3.4.3. Effect of α-MSH on PARP cleavage in SA-LPS-stimulated HMC-1 and BM-mast cells. Cells were stimulated with SA-LPS for 2 h and then treated with a-MSH (10 nM) for 36 h. Then cell extract proteins were assayed for PARP by Western blot.

dependent manner both in HMC-1 and BM-mast cells (Fig.3.4.3). α-MSH alone induced cell death in HMC-1 and BM-mast cells as detected by MTT assay and SA-LPS was unable to protect α-MSH-mediated cell death (Fig. 3.4.2) indicating the similar responses of α-MSH in human mast cell line and mouse bone marrow-derived mast cell as shown in MC-9 previously.

α-MSH inhibits NF-κB activation through cAMP generation

It has been reported that α-MSH transduces its signal through cAMP. To determine the role of cAMP, we used dideoxyadenosine (ddAdo), a potent inhibitor of adenylate cyclase (Dostmann, W.R, 1990), enzyme responsible for the generation of cAMP. Cells were exposed to SA-LPS (100 ng/ml) for 2 h followed by ddAdo (250 μM) for 1 h and then treated with α-MSH (10^6 pM) for 24 h. Then NF-κB assayed from nuclear extracts. The results shown in Figure 3.5.1 show that ddAdo did not interfere with SA-LPS-induced NF-κB activation, but it protected against α-MSH-mediated suppression of NF-κB stimulated by SA-LPS. Treatment of cells with exogenous cAMP (dibutyryl cAMP, 50μM) inhibited SA-LPS-induced NF-κB activation. (Fig.3.5.2). Since cAMP is known to activate PKA, we also examined the effects of two PKA inhibitors, Rp-cAMPS isomer and H8 and PKC inhibitor, H7 on the α-MSH-induced inhibition of NF-κB activation. For this, cells were stimulated with SA-LPS for 2 h followed by treatment with either Rp-cAMPS isomer (100 μM), H-8 (2 μM), or H7 (10 nM) for 1 h at 37°C and then treated with α-MSH for 24 h. NF-κB was assayed from nuclear extract. Pretreatment with both PKA inhibitors blocked the inhibitory effects of α-MSH, while PKC inhibitor H7 had no effect on α-MSH-mediated inhibition of NF-κB activation (Fig. 3.5.2), suggesting that the effect of the PKA in α-MSH-mediated action.

Upregulated NF-κB protects α-MSH-mediated cell death in MC-9 cells

In order to detect the role of NF-κB on α-MSH-mediated cell death, MC-9 cells were co-transfected with p65 plasmid and SEAP reporter DNA. As shown in Figure 3.6.1.1,

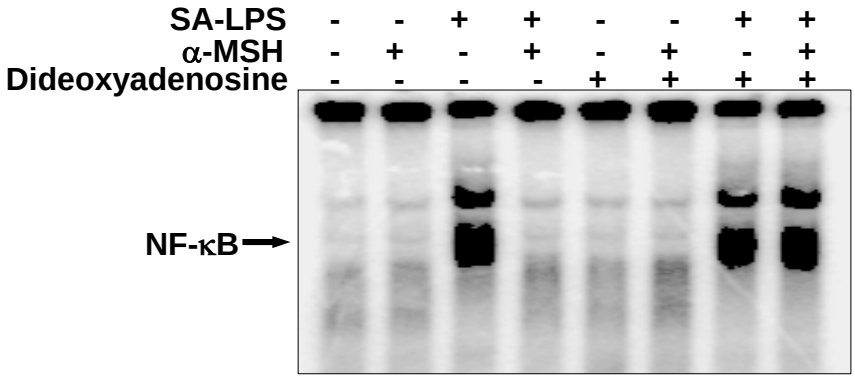


Fig 3.5.1 Role of cAMP in α-MSH induced NF-κB inhibition. ddAdo protects from α-MSH-mediated inhibition of NF-κB stimulated by SA-LPS. MC-9 cells ($2x10^6$/ml) were incubated with SA-LPS for 2 h followed by ddAdo (250 μM) for 1 h and then treated with α-MSH (10^6 pM) for 24 h. NF-κB was assayed from NEs.

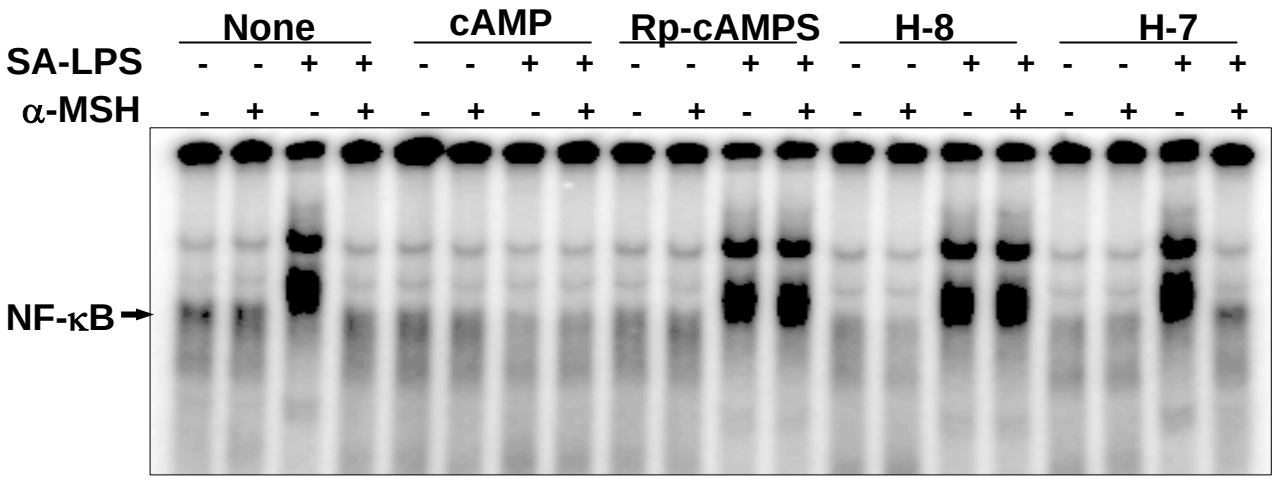


Fig.3.5.2. The PKA inhibitor, cAMP-Rp isomer and H8 protects α-MSH-mediated inhibition of NF-κB induced by SA-LPS. MC-9 cells, stimulated with SA-LPS for 2 h, were treated with dibutyryl cAMP (50 μM), Rp-cAMPS isomer (100 μM), H-8 (2 μM), or H7 (10 nM) for 1 h at 37^0C.Cells were then treated with α-MSH (10^6 pM) for 24 h. NEs were prepared and analyzed by EMSA.

p65 transfected MC-9 cells showed NF-κB activation and α-MSH treatment did not downregulate NF-κB. The SEAP activity was observed in p65 transfected MC-9 cells about 5-fold. α-MSH did not downregulate the SEAP activity similar to NF-κB activation (Fig.3.6.1.2 A). Non-transfected and p65 transfected cells were incubated with different concentrations of α-MSH for 36 h and the cytotoxicity was assayed by MTT uptake. The cell viability was decreased with increased concentrations of α-MSH in non-transfected MC-9 cells but p65 transfected cells showed about 10 % decrease of cell viability (Fig.3.6.1.2 B) indicating that NF-κB over-expressed cells are resistant to α-MSH-mediated apoptosis.

Downregulation of NF-κB by IκBα-DN does not induce cell death in HuT-78 cells

To confirm the role of NF-κB on α-MSH-mediated cell death, HuT-78 cells (constitutively activated with NF-κB) were transfected with IκBα-DN construct (mutation on Ser^{32} and Ser^{36} position) and then these transfected and non-transfected cells were treated with α-MSH for 36 h. NF-κB was assayed from nuclear extracts. As shown in figure 3.6.2.1, α-MSH-mediated downregulation of NF-κB (high activity of basal level) was not observed. 50-fold cold oligo suppressed the band indicating its specificity. In IκBα-DN transfected cells, no NF-κB activity was observed. The SEAP activity from culture supernatant showed that the high basal level (4-fold) was not decreased by α-MSH. IκBα-DN transfected cells did not show much SEAP activity (Fig.3.6.2.2 A). In order to detect the role of NF-κB on α-MSH-mediated cell death, HuT-78 cells non-transfected and transfected with DN-IκBα were treated with different concentrations of α-MSH for 36 h and then cell viability was assayed by MTT uptake. HuT-78 cells showed 10-20 % decrease in cell viability but DN-IκBα transfected cells showed cell death in a dose dependent manner by α-SH (Fig.3.6.2.2 B) indicating involvement of NF-κB in α-MSH-mediated cell death.

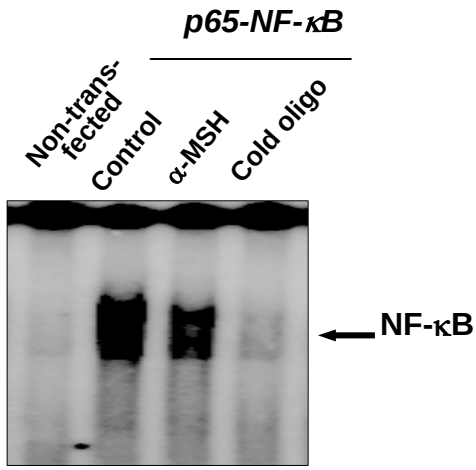


Fig 3.6.1.1 Overexpressed NF-κB protects α–MSH-mediated inhibition of NF-κB and apoptosis in MC-9 cells. MC-9 cells were transfected with or without p65 linked with SEAP construct, cultured for 12 h, and then treated with α-MSH for 24 h. The NF-κB was assayed from NEs using 50-fold cold oligo for specifficity.

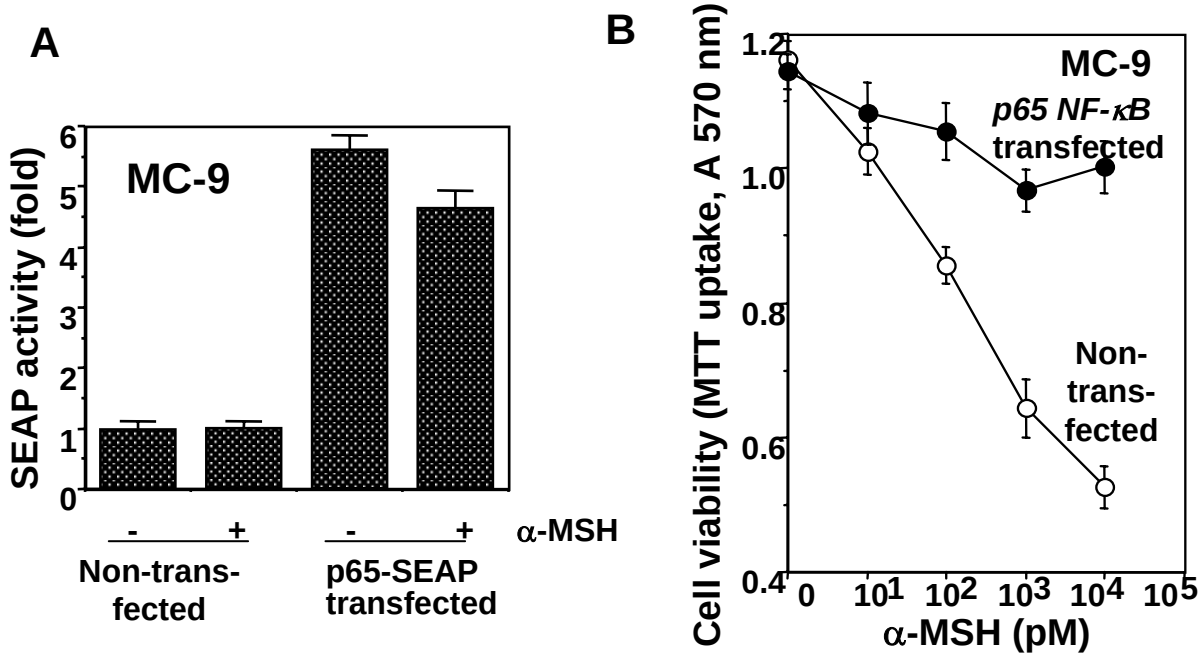


Fig 3.6.1.2 Overexpressed NF-κB protects α–MSH-mediated inhibition of NF-κB and apoptosis in MC-9 cells. **A**. MC-9 cells were transfected with or without p65 linked with SEAP construct, cultured for 12 h, and then treated with α-MSH for 24 h and SEAP activity was assayed from cultured conditioned medium. **B.** Transfected or non transfected cells were then treated with different concentrations of α-MSH for 36 h and assayed for cell viability by MTT uptake.

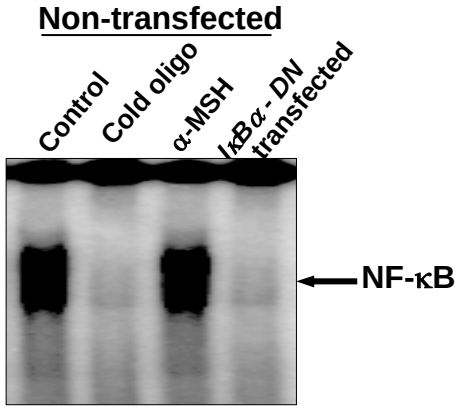


Fig.3.6.2.1 Effect α-MSH on HuT-78 cells transfected with IκBα-DN construct. HuT-78 cells were transfected with or without IκBα-DN construct and cultured for 12 h. Then cells were treated with α-MSH for 24 h and NF-kB was assayed from the NEs.

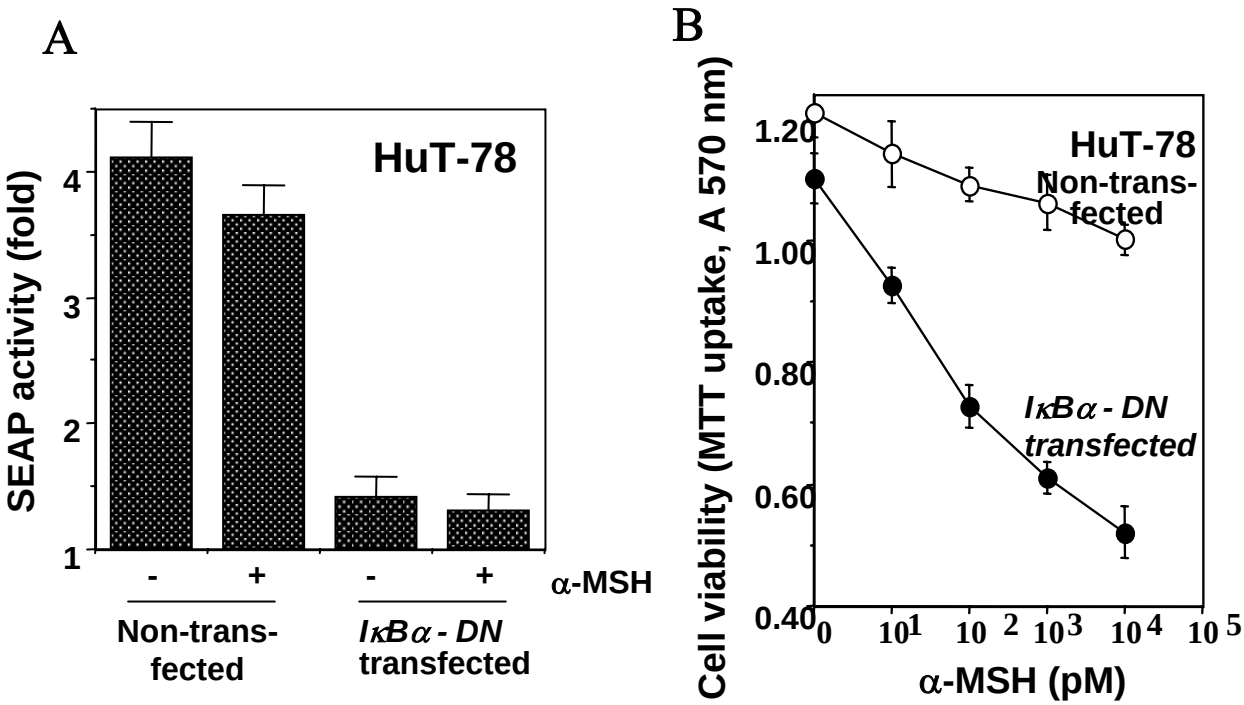


Fig.3.6.2.2 Effect α-MSH on HuT-78 cells transfected with IκBα-DN construct. HuT-78 cells were transfected with with or without IκBα-DN construct and cultured for 12 h. Then cells were treated with α-MSH for 24 h . **A.** SEAP activity was assayed from cultured conditioned medium. **B.** Non-transfected and IκBα-DN-transfected HuT-78 cells were treated with different concentrations of α-MSH for 36 h and cell viability was assayed**.**

3.3 Discussion

Even though several studies indicate that certain neuropeptides, such as α-MSH, have anti-inflammatory effects, the mechanism underlying this effect is not understood. Mast cells are quick responders in allergic and inflammatory diseases. As inflammatory responses are aggravated by NF-κB activation followed by NF-κB-dependent gene activation, our strategy was to regulate this transcription factor. In this report we observed that endotoxin induced NF-κB and NF-κB-dependent reporter gene activation was inhibited by α-MSH (Figs .3.1.1, 3.1.4, 3.1.5). α-MSH binds with its receptor predominantly with melanocortin-1 receptor. The level of α-MSH receptors as detected by radiolabeled α-MSH binding (Fig.3.2.1) and Western blot (Fig.3.2.2) reflects the α-MSH-mediated NF-κB inhibition. The viable cell number as detected by MTT dye uptake (Fig.3.3.1) and proliferating cell number by thymidine incorporation (Fig.3.3.2) suggests that α-MSH treatment led to cell death. Lipid peroxidation (Fig.3.3.3 A), ROI generation (Fig.3.3.3 B), precursor caspase 8 cleavage (Fig.3.3.4), and caspase-dependent PARP protein cleavage (Fig.3.3.5) all supported cell death. SA-LPS has been reported to maintain NF-κB activation for a long time (Manna and Aggarwal, 1999), which may be due to ROI generated by it. However, an additional generation of ROI by α-MSH may be detrimental to the cells. α-MSH-mediated biological activities were observed not only in murine mast cell line but also in human mast cell line and mouse bone marrow-derived primary mast cells. The reports so far available were that α-MSH down-regulates TNF-induced NF-κB activation through generation of cAMP and activation of PKA (Manna and Aggarwal, 1998). How generated cAMP inhibits NF-κB activation was also investigated. It was also shown that the catalytic subunit of PKA (PKAc) associates with IκB-α, the inhibitory subunit of NF-κB in the cytoplasm (Zhong *et al.,* 1997). A lot of genes are upregulated including IκB-α, upon activation of NF-κB. This IκBα possibly binds with catalytic subunit of PKA forming PKAc-IκBα-p65 (NF-κB) complex thereby inhibiting NF-κB activation. How cAMP downregulates NF-κB remains to be elucidated

and ddAdo, adenylate cyclase inhibitor reverses the function. cAMP induces transcription factors like AP-1 and CREB, but not NF-κB (Hershko *et al.*, 2002). NF-κB may be negatively regulated by CREB. Downregulation of NF-κB reflects on cell death as shown in α-MSH-treated cells. Surprisingly, α-MSH did not downregulate NF-κB in mast cells overexpressed with p65 (NF-κB) or HuT-78 which possess constitutively activated NF-κB and showed 10-20 % induction of cell death (Fig.3.6). Mechanism of endotoxin-mediated NF-κB activation may be different than constitutively expressed NF-κB in HuT-78 cells or overexpressed with p65 (NF-κB) in MC-9 cells. From these results it is clear that α-MSH-mediated induction of cell death in mast cells depends on NF-κB.

As mast cells are key responder of allergic and inflammatory responses in asthma, gout arthritis, rheumatoid arthritis etc and basal activation of NF-κB has been shown in these diseases, so induction of cell death by α-MSH in mast cells by downregulating NF-κB will be helpful to regulate cell number in these diseases.

CHAPTER FOUR

ROLE OF

α-MELANOCYTE STIMULATING HORMONE

IN MACROPHAGES

4.1 Introduction

Sepsis is a major complication observed after major surgery in the intensive care units. Bacterial lipopolysaccharide (LPS), a component of Gram-negative bacteria that sheds into circulation is the major source of sepsis. LPS can initiate hypermetabolic shock termed as endotoxic shock, collapsing different organ co-ordination leading to multiple organ dysfunctions (Meakins JL, 1990). LPS mediated biologic manifestations involves activation of several kinases, and transcription factors followed by an uncontrolled production and release of proinflammatory cytokines such as TNF, IL-1, IL-6, IL-8, IL-10, IL-12 etc, reactive oxygen species, and proteolytic enzymes (Mackman *et al.*, 1991; Vincenti *et al.*, 1992; Sanlioglu *et al.*, 2001) by cells. LPS interacts with most cells through CD14, a 55-kDa glycophosphatidylinositol (GPI)-anchored membrane protein expressed on the surface of monocytes, macrophages, and neutrophils (Haziot *et al.*, 1998; Wright *et al.*, 1990)

Monocytes/macrophages are the first cells that are involved in inflammation. Receptors for α-MSH have been detected in different cell types (Catania *et al.*, 1996; Star *et al.*, 1995), including macrophages. Alpha-Melanocyte-stimulating hormone (α-MSH) exerts its anti-inflammatory actions induced by a variety of stimuli, by down-regulating either the production or the action of the proinflammatory cytokines mainly via the modulation of NF-κB pathway (Catania A, 1996; Rajora *et al.*, 1996; Mandrika *et al.*, 2001; Watanabe *et al.*, 1993; Manna and Aggarwal, 1999). In mouse macrophages, α-MSH has been shown to inhibit the induction of C/EBPβ DNA binding activity and that this effect is a major mechanism by which α-MSH inhibits the transcription of the NOS2 gene (Gupta *et al.*, 2001). As endotoxin mediated activation of inflammatory responses and septic shock was suppressed by α-MSH, the detail mechanism of effect of α-MSH on LPS mediated responses was investigated.

4.2 Results

In this study, we examined the effect α-MSH on SA-LPS-induced biological responses. Non-adherent THP-1 monocytic cells were converted into adherent macrophages by PMA (10 ng/ml) in 16 h and these cells were induced with SA-LPS (100 ng of LPS was incubated with 20 μl of human serum for 1 h at 37^0C and this mixture was SA-LPS).

α-MSH inhibits myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase activity -induced by SA-LPS.

To detect the role of α-MSH in SA-LPS-mediated biological responses in macrophages, cells were incubated with α-MSH (100 nM) for 24 h and then stimulated with different concentrations of SA-LPS for 2 h at 37^0C. Culture supernatant was then used to analyze myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase (Fig.4.1.1). The absorbance for myeloperoxidase and β-D-glucuronidase, but not that of alkaline phosphatase, was enhanced with increasing concentrations of SA-LPS and α-MSH pre-treatment inhibited their activity. The results are mean OD of triplicate assays. From these results it is clear that SA-LPS mediated proteolytic enzymes release is inhibited by α-MSH.

α-MSH inhibits oxidative burst response induced by SA-LPS-, but not FMLP.

LPS and formyl peptide (FMLP) are reported to induce generation of reactive oxygen species (ROS) from macrophages (Manna *et al.*, 1997), which is detected by Nitro blue tetrazolium test (NBT). To detect the role of α-MSH on SA-LPS-mediated ROS generation, macrophages were treated with different concentrations of α-MSH for 24 h and then stimulated with 100 ng/ml SA-LPS or 100 nM FMLP, for 2 h at 37^0C, in presence of NBT solution (0.1%). NBT positive cells were counted under microscope and presented in percentage above unstimulated cells (Fig.4.1.2). α-MSH did not increase the number of NBT positive cells on its own in the time and dose used in this experiment. SA-

LPS and FMLP induced 82 % NBT positive cells. α-MSH inhibited SA-LPS-induced NBT positive cells but not FMLP-induced. These data indicate that α-MSH down-regulates SA-LPS, but not FMLP-induced oxidative burst response suggesting the specific activity of α-MSH.

α-MSH inhibits nitric oxide production induced by SA-LPS.

To detect the role of α-MSH on SA-LPS-induced NO production, macrophages were treated with α-MSH (100 nM) for 24 h and then stimulated with different concentrations of SA-LPS for 4 h at 37^0C. The culture supernatant was collected and then analyzed for NO using Griess reagent. The concentration of NO was determined from the standard curve obtained from the reaction of $NaNO_2$. The results indicated in Figure 4.1.3A reveal that SA-LPS induced NO production in a dose dependent manner, which is inhibited by treatment with α-MSH.

α-MSH inhibits SA-LPS-induced ROI generation.

ROI generation is an intermediate step for different biological responses. To detect the role of α-MSH on SA-LPS-induced ROI generation, macrophages were treated with α-MSH (100 nM) for 24 h and then stimulated with different concentrations of SA-LPS for 4 h. Then ROI generation was examined with dihydrorhodamine dye conversion to rhodamine as described in materials and methods. The results indicate that α-MSH inhibited SA-LPS-induced ROI generation (Fig.4.1.3 B).

α-MSH inhibits activation of NF-κB, induced by SA-LPS.

To detect the role of α-MSH on activation of NF-κB induced by endotoxin, macrophages were treated with different concentrations of α-MSH for 24 h 37^0C and then stimulated with 100 ng/ml SA-LPS for 2 h at 37^0C. Nuclear extract (NE) was prepared and 8 μg NE proteins were analyzed in 6.6% native PAGE to detect NF-κB by gel shift

Table-1: Specific ^{125}I-α-MSH binding to cells

Cells	Specific binding ± SD (cpm)
H4	1340 ± 120
U-937	1230 ± 102
THP-1	1672 ± 214
Macrophages	3020 ± 230
Jurkat	2140 ± 210
HeLa	858 ± 92
NIH 3T3	812 ± 152
Neutrophils	2678 ± 156

Table 1. Detection of α-MSH receptors in different cells. Different cells were incubated with ^{125}I-α-MSH for 2 h at 4^0C in presence or absence of 50-fold unlabeled α-MSH. The specific α-MSH binding was assayed after subtracting the non-specific binding (binding in presence of 50-fold cold α-MSH) from total binding (binding in absence of cold α-MSH). The results indicated as mean specific binding (in cpm) of triplicate assays.

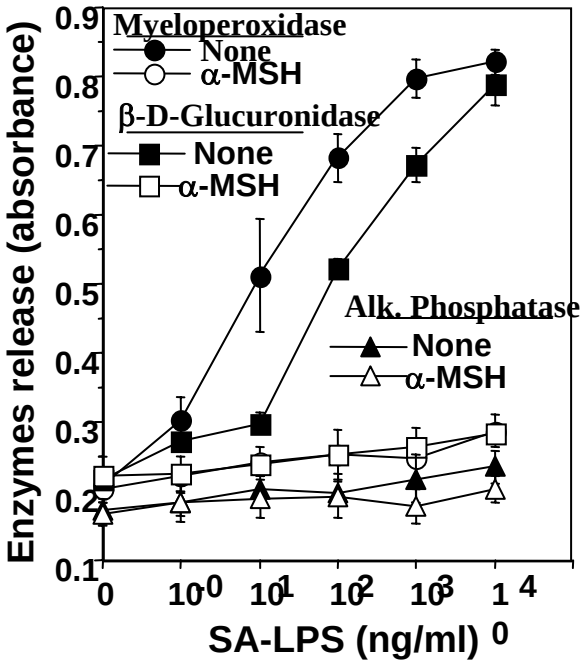


Fig.4.1.1. Effect of α-MSH on SA-LPS-induced enzymes release. Macrophages were treated with 100 nM α-MSH for 24 h and then stimulated with different concentrations of SA-LPS for 2 h. The supernatant was then analyzed for myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase.

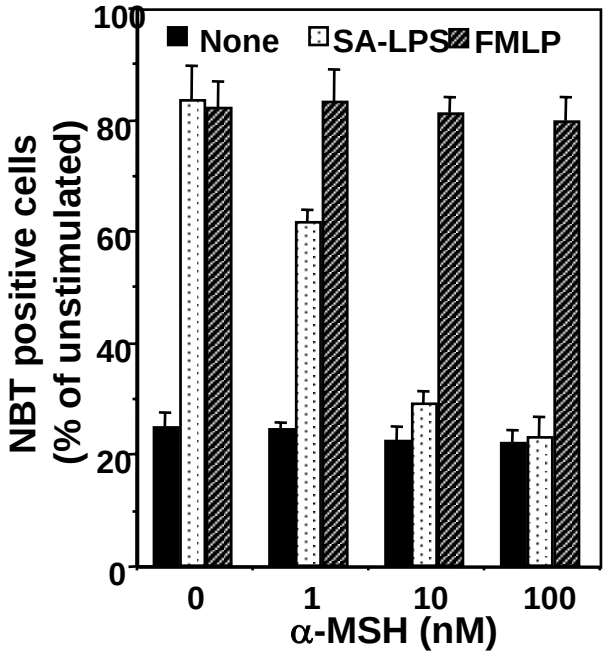


Fig. 4.1.2 Effect of α-MSH on SA-LPS- and FMLP-induced oxidative burst response. Cells were treated with α-MSH for 24 h, stimulated with 100 ng/ml SA-LPS or 100 nM FMLP for 2 h. Then NBT test was carried out. NBT positive and negative cells were counted under microscope and positive cells were presented in percentage.

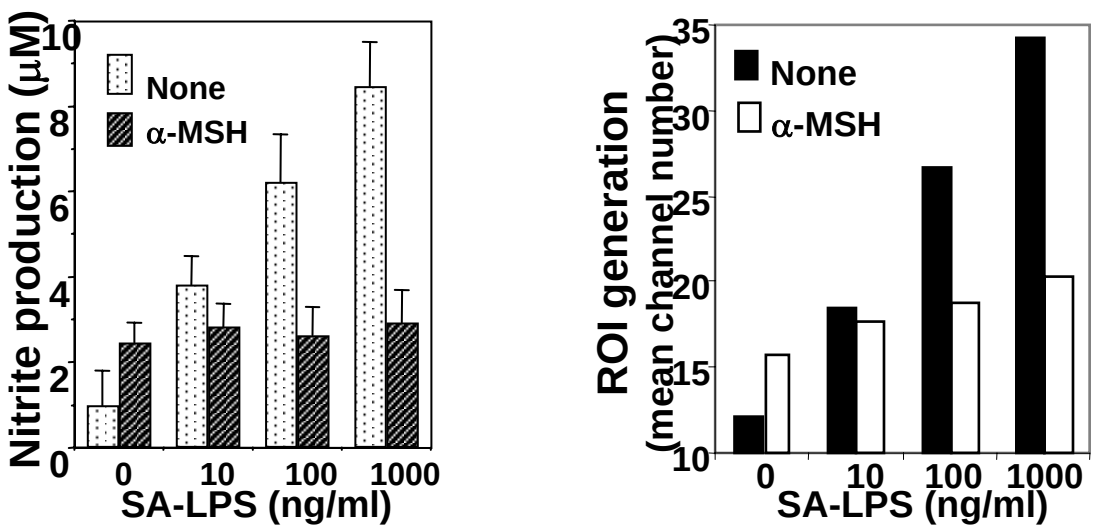


Fig. 4.1.3. Effect of α-MSH on SA-LPS-induced NO and ROI generation. Macrophages were treated with 100 nM α-MSH for 24 at 37^0C and then stimulated with different concentrations of SA-LPS for 4 h. **A.** The nitrite concentration in the supernatants was then measured as described under Materials and methods. **B.** ROI generation was assayed as mean channel number by dihydrorhodamine in Flow cytometer.

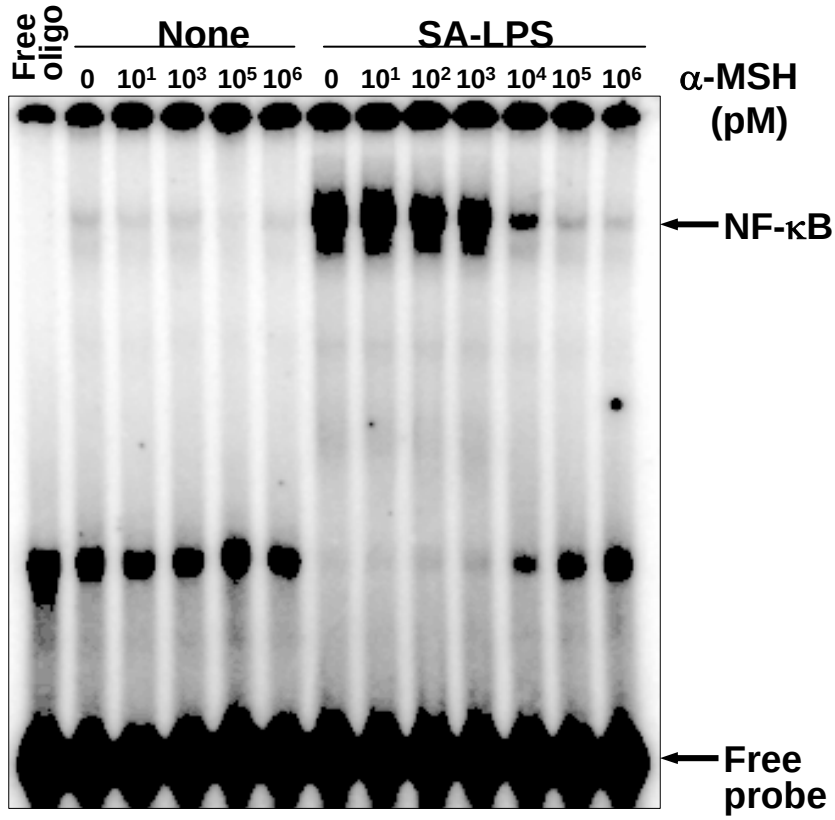


Fig. 4.2.1. Effect of α-MSH on SA-LPS-induced NF-κB activation. Macrophages (2 x 10^6/ml) were treated with different concentrations of α-MSH for 24 h at 37^0C, CO_2 incubator and then stimulated with SA-LPS (100 ng/ml) for 2 h. NF-κB was assayed from 8 μg NE proteins.

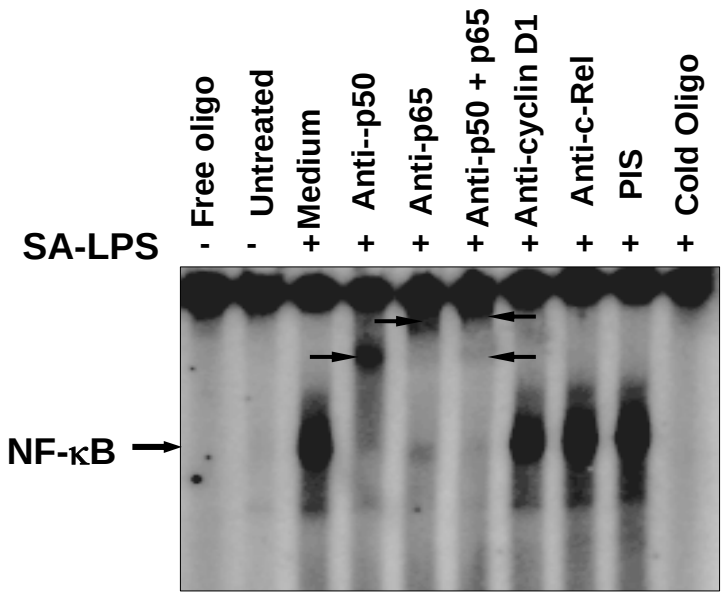


Fig. 4.2.2. Supershift of NF-κB band. SA-LPS-induced NE was incubated for 15 min with different antibodies and cold NF-κB oligonucleotide and then assayed for NF-κB.

assay. The results in Figure 4.2.1 indicate that, α-MSH alone did not activate NF-κB, but inhibited SA-LPS-induced NF-κB activation in a dose dependent manner. Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. To show specificity of the retarted band as NF-κB, we incubated the nuclear extracts from SA-LPS-induced cells with antibodies (Abs) for p50 (NF-κBI) and p65 (Rel A) or in combination and then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher molecular size position (Fig.4.2.2), thus suggesting that the SA-LPS-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant Abs such as anti-c-Rel or anti-cyclin D1 had any effect on the mobility of NF-κB. The complex completely disappeared in the presence of 50-fold molar excess of cold NF-κB indicating the specificity of NF-κB.

α-MSH inhibits SA-LPS-induced activation of NF-κB reporter gene.

As SA-LPS-induced activation of NF-κB was blocked by α-MSH, the NF-κB dependent gene expression was also carried out. Macrophages were transfected with NF-κB reporter plasmid containing SEAP (secretory alkaline phosphatase) gene with or without dominant negative IκBα (IκBα-DN) plasmid. Cells were treated with different concentrations of α-MSH for 24 h at 37^0C and then stimulated with SA-LPS for 4 h. Culture supernatant was collected and used to assay activity of SEAP. The results in Figure 4.2.3 A indicate that SEAP activity was induced by SA-LPS. α-MSH alone had no effect on SEAP activity but inhibited SA-LPS-induced SEAP in a dose-dependent manner. The dominant negative IκBα transfected cells showed the basal activity of SEAP and SA-LPS-mediated stimulation was completely abrogated by the cells transfected with IκBα-DN (Fig.4.2.3 B) indicating the specificity of the assay.

α-MSH inhibits expression of ICAM-1, induced SA-LPS.

As α-MSH inhibited SA-LPS-induced different biological responses, the expression of adhesion molecule, ICAM-1 was analyzed. Macrophages were treated with α-MSH (100 nM) for 24 h and then stimulated with different concentrations of SA-LPS for 12 h.

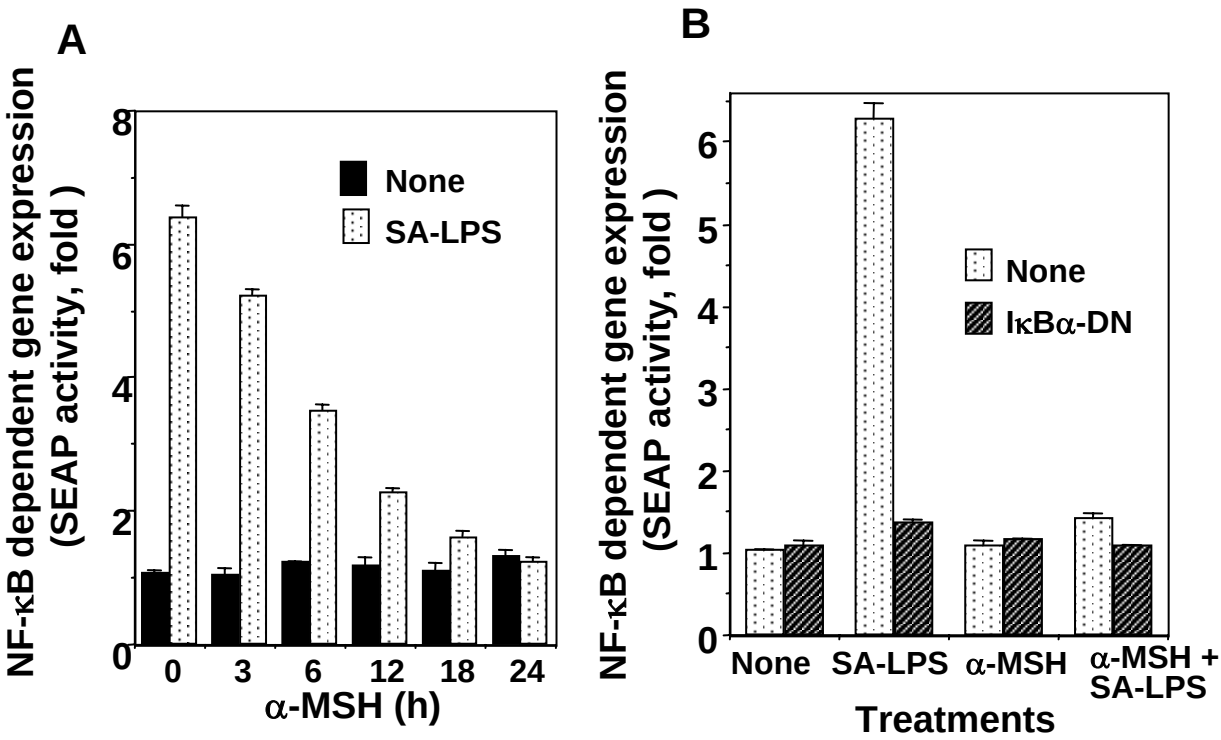


Fig.4.2.3. Effect of α-MSH on SA-LPS-induced NF-κB-dependent reporter gene expression. A. Macrophages were transiently transfected with NF-κB-containing plasmid linked to the SEAP gene. **B.** Macrophages were transiently transfected with IκBα-DN plasmid along with NF-κB-containing plasmid linked to the SEAP gene. In both sets of experiments, the cells were cultured for 12 h, treated with 100 nM of α-MSH for different times and then stimulated with SA-LPS for 4 h. Culture supernatant was taken and assayed for SEAP.

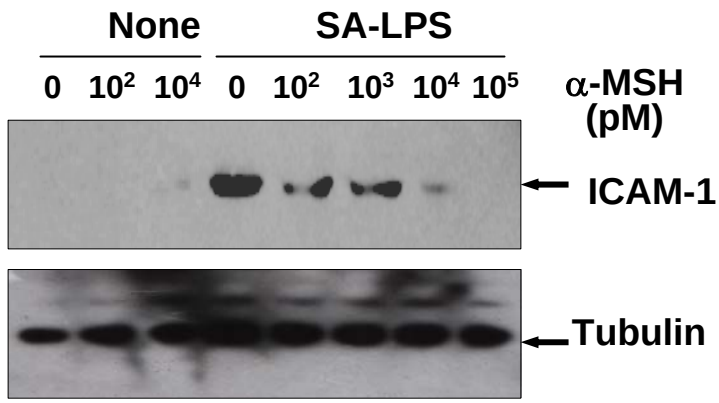


Fig. 4.2.4. Effect of α-MSH on SA-LPS-induced ICAM-1 expression. Macrophages, either untreated or α-MSH (100 nM) pre-treated for 24 h were stimulated with different concentrations of SA-LPS for 12 h. Then cell extract proteins were assayed for ICAM1 by Western blot. Same blot was re-probed to detect tubulin by Western blot to show equal loading of extract proteins.

Then 100μg cell extract proteins were analyzed in 9% SDS-PAGE for ICAM-1 using Western blot technique. The Figure 4.2.4 show that SA-LPS induced expression of ICAM-1 in a dose-dependent manner but α-MSH pretreated cells inhibited it suggesting inhibition of SA-LPS-induced biological responses in macrophages, by α-MSH. Upon re-probing the gel with anti-tubulin antibody, we found that the band intensities in all lanes were uniform indicating equal loading of extracted protein in the lanes.

Anti-MC-1R Ab protects α-MSH-mediated decrease in NF-κB activation induced by SA-LPS.

To detect the specificity of α-MSH mediated inhibition of SA-LPS-induced biological responses, macrophages ($1x10^6$/well in 6-well plate) were incubated with 1 μg of anti-melanocortin 1 receptor (anti-MC-1R) antibody for 2 h and then treated with α-MSH (100 ng/ml) for 24 h. Subsequently, cells were stimulated with 100 ng/ml of SA-LPS for 2 h. Nuclear extracts were prepared and 8 μg nuclear proteins were analyzed for NF-κB by gel shift assay. Figure 4.3.1 shows that SA-LPS-induced NF-κB activation was inhibited by α-MSH. α-MSH treated cells did not inhibit SA-LPS-induced activation of NF-κB when cells were pre-incubated with anti-MC-1R Ab indicating that α-MSH exerts its function via MC-1R.

Polymixin B sulphate blocks SA-LPS-induced NF-κB activation.

LPS is a glycolipid, and its lipid moiety is known to be critical for its activity. Polymyxin B, a polycationic cyclic peptide, is known to bind to the lipid moiety of LPS and inactivate its activity (Srimal *et al.,* 1996). To ascertain this exquisite specificity for NF-κB activation, we incubated 10 μg of polymixin B sulphate with SA-LPS for 1 h at 37^0C and then this mixture was added to macrophages for 2 h at 37^0C. Nuclear extracts were prepared and assayed for NF-κB by EMSA. The results in Fig. 4.3.2 show that polymyxin B by itself had no effect on NF-κB activation, but it completely abrogated SA-LPS-induced NF-κB activation.

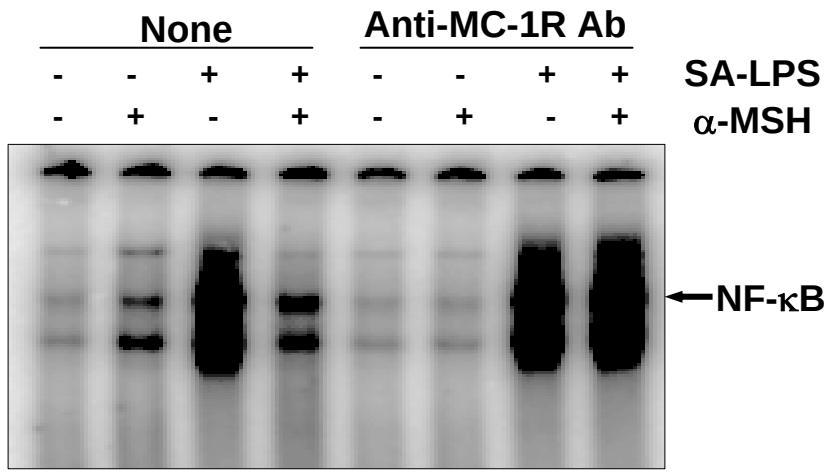


Fig.4.3.1 Effect of anti-MC-1R antibody on α-MSH-mediated downregulation of SA-LPS-induced NF-κB activation. Macrophages, pre-incubated with anti-MC-1R Ab (1μg/ml) for 2 h were treated with 100 nM α-MSH for 24 h. Cells were then stimulated with 100 ng/ml SA-LPS for 2 h. NF-κB was assayed from nuclear extract.

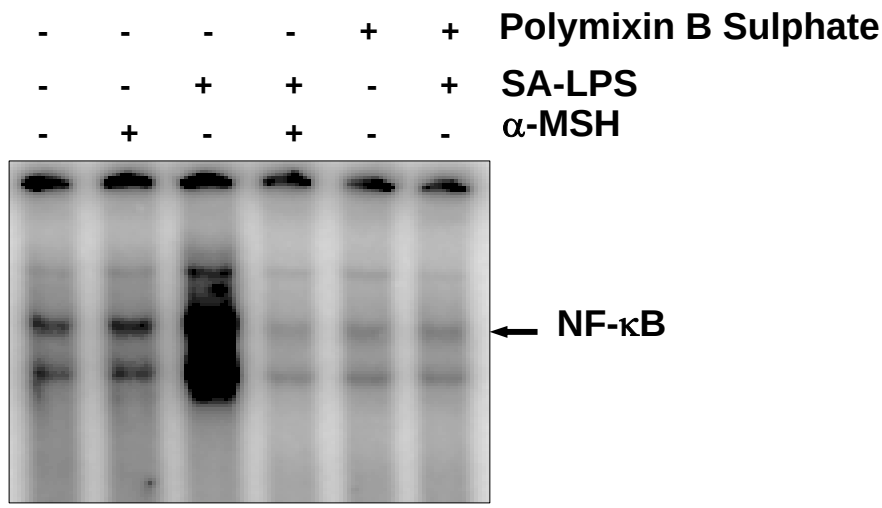


Fig. 4.3.2. Effect of polymixin B-sulphate on SA-LPS-induced NF-κB activation. 10 μg of polymixin B sulphate was incubated with SA-LPS for 1 h at 37^0C and then this mixture was added to macrophages for 2 h at 37^0C. Nuclear extracts were prepared and assayed for NF-κB by EMSA.

Inhibition of NF-κB activation by α-MSH is cell type specific.

As NF-κB activation pathways differ in many cell types (Bonizzi *et al.,* 1997; Li and Karin, 1998; Imbert *et al.,* 1996; Dostmann *et al.,* 1990). Although all the experiments in this part of the work were carried out in macrophages, we also studied whether α-MSH affects human Jurkat (T) cells and H4 (glioma) cells and neutrophils isolated from fresh human blood as well. It has been demonstrated that distinct signal transduction pathways could mediate induction in lymphoid, neuronal, and primary cells. We found that α-MSH blocks SA-LPS-induced NF-κB activation in neutrophils (Fig.4.4.A) and Jurkat cells (Fig. 4.4.B) in a dose-dependent manner, but partially in H4 cells (Fig.4.4.C) suggest that this effect of α-MSH is restricted to neutrophils and T-cells, but not glioma cells.

α-MSH downregulates CD14, but not for TNFR1, TNFR2 or IL-1R1, in macrophages.

To detect the role of α-MSH on the level of different receptors, macrophages were treated with 100 nM of α-MSH for different time points. Then cells and culture supernatant (concentrated 10 times) were used to assay TNFR1, TNFR2, IL-1R1, or CD14 using ^{125}I-IgG binding assay as described in materials and methods. The Figure 4.5 show that specific binding for TNFR1 (Fig.4.5.1 A), TNFR2 (Fig.4.5.1 B), or IL-1R1 (Fig.4.5.1 C) was not changed with varying time of α-MSH treatment both in cells and culture supernatant. However, the specific binding of ^{125}I-IgG for CD14 was decreased from cells with increasing time of α-MSH treatment and started appearing in the culture supernatant (Fig.4.5.1 D). The level of TNFR1 was unchanged in the cell extracts treated with α-MSH with no detectable level in the culture supernatant as assayed by Western blot (Fig.4.5.2 A). The level of CD14 decreased in the cell extract with increasing time of α-MSH treatment with a corresponding increase in the culture supernatant (Fig.4.5.2 B) supporting the results obtained in the binding assays. Overall, the results indicate that, α-MSH induces release of CD14 from surface of macrophage but not affect other receptors under study (IL-1R, TNFR1, and TNFR2). As loading control the same blot was reprobed for alkaline phosphatase (Fig. 4.5.3).

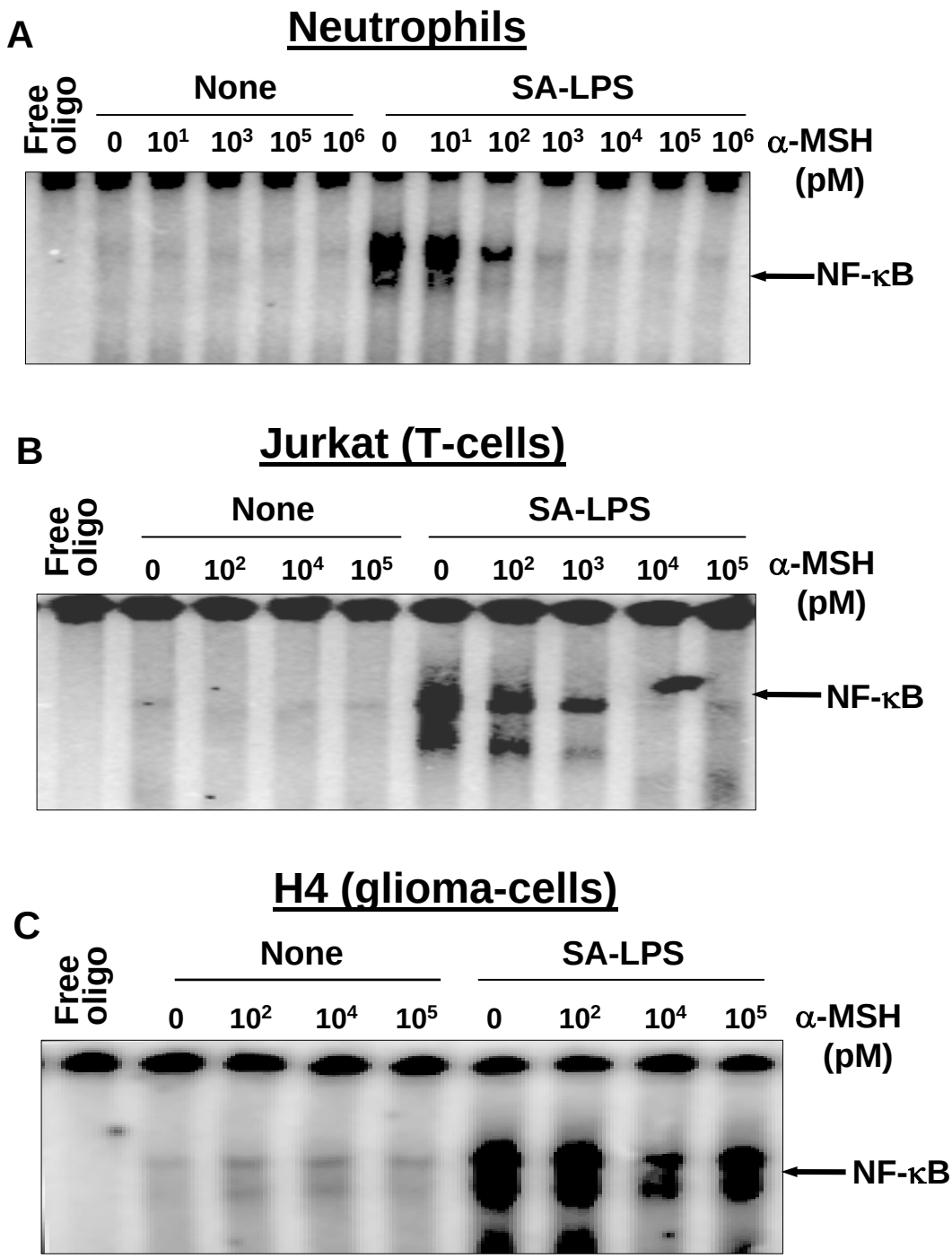


Fig.4.4. Effect of α-MSH on SA-LPS-induced NF-κB activation in different cells. human neutrophils isolated from fresh human blood. Cells (**A**), Jurkat (Human T cells) (**B**) and H4 glioma cells (**C**) were used for the study. Cells were treated with different concentrations of α-MSH for 24 h and then stimulated with SA-LPS (100 ng/ml) for 2 h at 37°C. After these treatments, nuclear extracts were prepared and assayed for NF-κB.

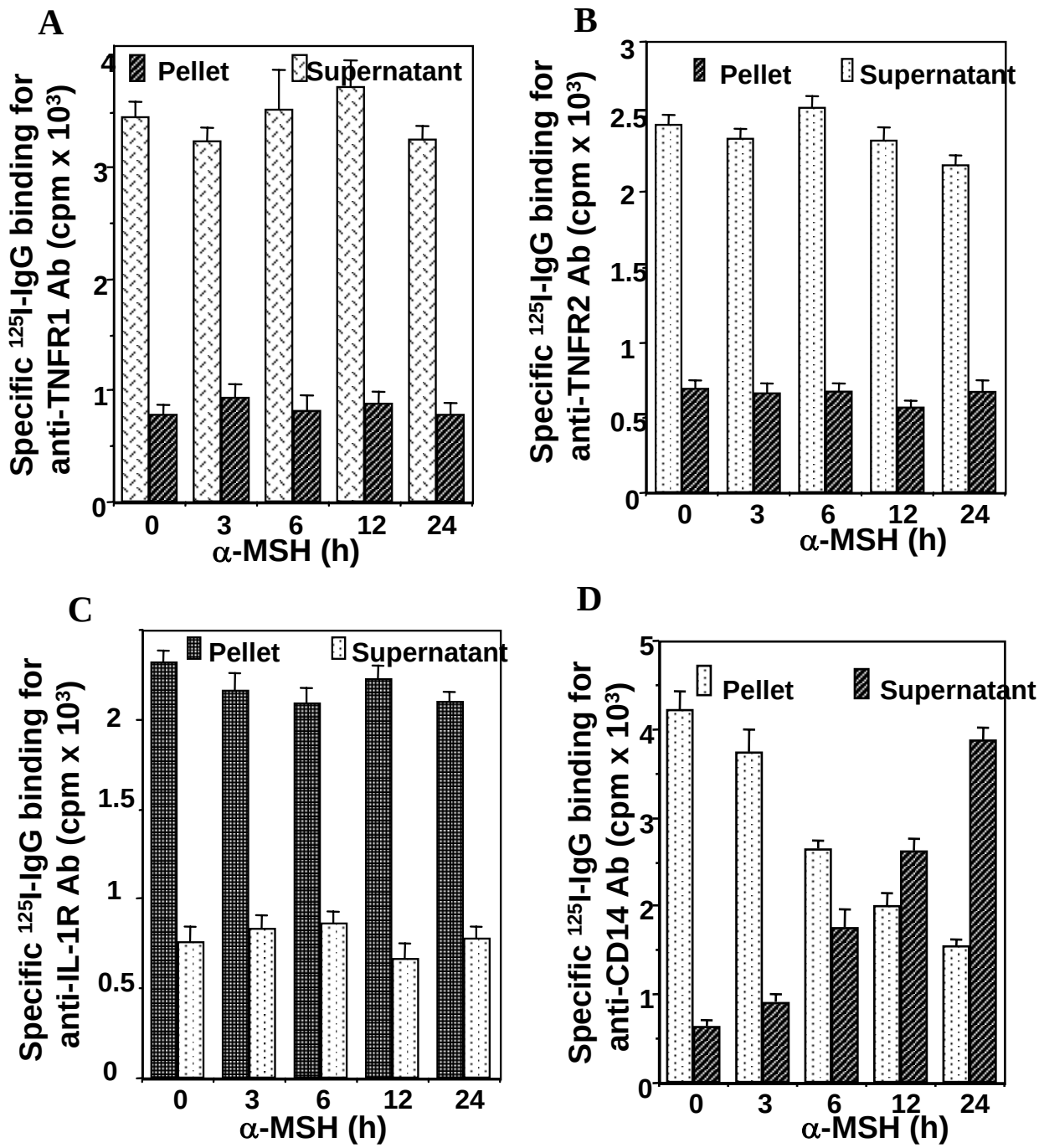


Fig.4.5.1. Effect of α-MSH level of different receptor . Macrophages were treated with 100 nM α-MSH for different times. After treatment, cells and 10 times concentrated culture supernatants (taken in nitrocellulose membrane disc) were incubated with **A.** anti- TNFR1 Ab, **B** anti-TNFR2 Ab, **C.** anti-IL-1R Ab and **D.** anti-CD14 Ab. Then specific ^{125}I-IgG (mouse) binding was assayed as described in Materials and methods.

α-MSH downregulates CD14 by secretion from macrophages cell surface.

To detect the mode of CD14 downregulation by α-MSH treated macrophages, cells were treated with α-MSH (100 nM) for 24 h and then cell supernatant, plasma membrane and cytoplasm (which includes cell organelles) was examined. Cell supernatant (100 μg proteins, after 10 times concentrated), plasma membrane and cytosol (from $1x10^6$ cells) were solubilized in SDS-PAGE sample buffer and then analyzed for CD14 by Western blot analysis. The bands were detected by ECL^{Plus} reagent (Amersham, IL) and detected in PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The level of CD14 was observed in membrane fraction of unstimulated cells and in culture supernatant in α-MSH treated cells (Fig.4.6.1). The CD14 was not observed in cytosolic fraction.

Anti-MC-1R Ab protects α-MSH-mediated downregulation of SA-LPS-induced NF-κB activation.

To detect the role of melanocortin receptor on α-MSH-mediated downregulation of CD14, macrophages were incubated with anti-MC-1R Ab (1 μg/ml) for 2 h and then treated with α-MSH (100 nM) for 24 h. Then cells supernatant was collected and concentrated (10 times). The cell pellet was extracted and 100 μg extract proteins and 50 μg cell supernatant proteins were analyzed in 10% SDS-PAGE and detected for CD14 by Western blot analysis. The result in Figure 4.6.2 indicates that α-MSH decreased CD14 level from macrophages cell surface and released it into the culture supernatant.

Anti-CD14 Ab does not protect α-MSH-mediated downregulation of CD14 and NF-κB activation.

To detect the role of anti-CD14 Ab on α-MSH-mediated CD14 downregulation, macrophages were incubated with anti-CD14 Ab (1 μg/ml) for 2 h and then treated with different concentrations of α-MSH for 24 h. Cells were then washed with 0.5 M potassium thiocyanate (KSCN) for 10 sec. After immediate washing, cells were incubated with anti-CD14 Ab for 2 h followed by ^{125}I-IgG binding at 37^0C for 1 h. The specific

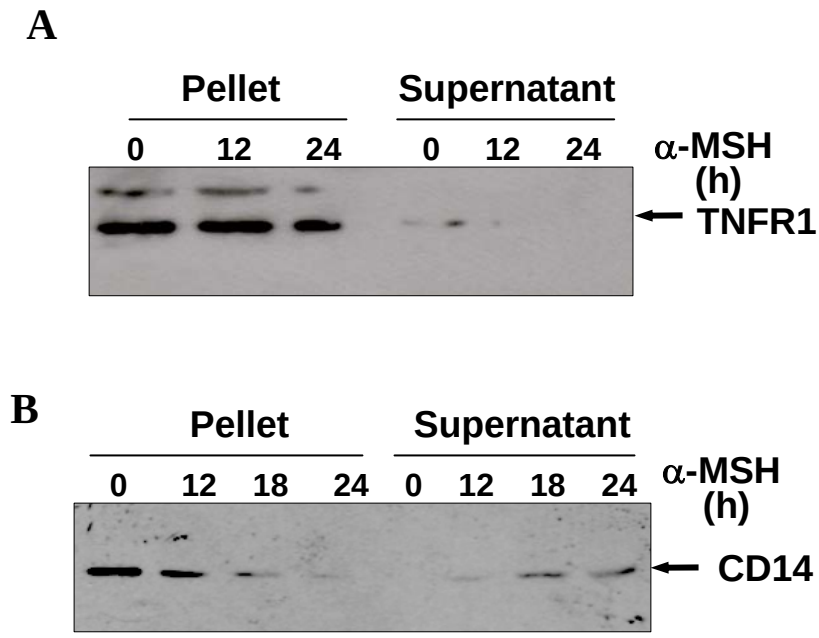


Fig. 4.5.2 Effect of α-MSH on binding of anti-TNFR1 and CD14 Ab binding. Macrophages were treated with 100 nM α-MSH for different times. After treatment, Cell pellet was extracted and 100 μg of extract proteins and 50 μg of concentrated culture supernatant proteins were assayed for TNFR1 (**A**) and CD14 (**B**) by Western blot analysis.

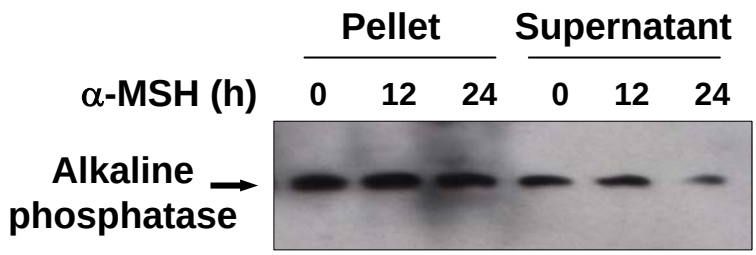


Fig. 4.5.3. The above blots were stripped and reprobed with anti- Alkaline phosphatase antibody to shown equal loading of proteins.

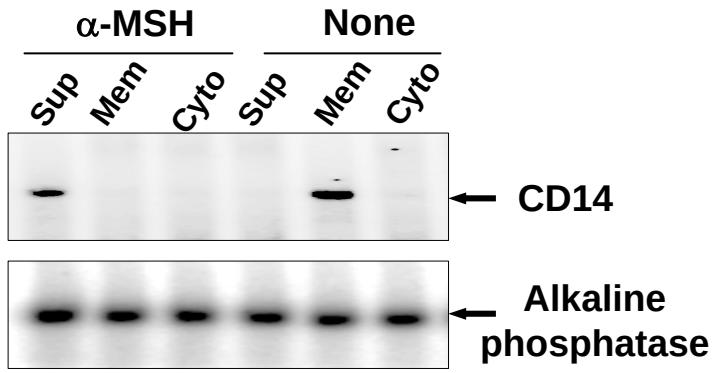


Fig. 4.6.1. Effect of α-MSH on downregulation of CD14. Macrophages were stimulated with α-MSH (100 nM) for 24 h and then culture supernatant (Sup) was concentrated 10 fold and 100 μg protein was taken in a tube. The plasma membrane (Mem) and cytoplasm (Cyto) was fractionated from untreated and α-MSH-treated cells (1 x 10^6 cells). The sup, membrane and cytosol were boiled in SDS-PAGE sample buffer. CD14 was detected by Western blot. Same blot was re-probed for alkaline phosphatse by Western blot analysis and bands were detected by chemiluminescence.

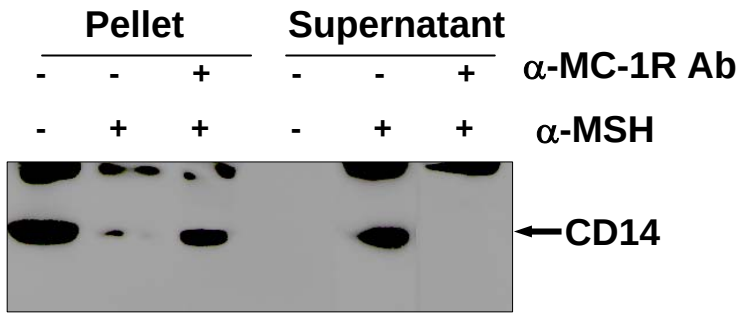


Fig. 4.6.2. Effect of anti-MC-1R antibody on α-MSH-mediated downregulation of CD14. Macrophages, pre-incubated with anti-MC-1R Ab (1μg/ml) for 2 h were treated with 100 nM of α-MSH for 24 h. The cells extract proteins (100 μg) and concentrated culture supernatant proteins (50 μg) were analyzed in 10% SDS-PAGE to detect CD14 by Western blot analysis.

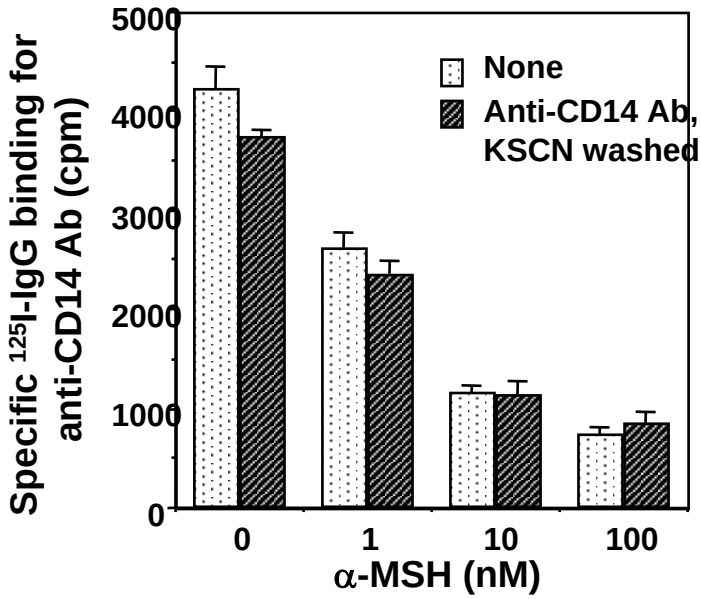


Fig. 4.6.3. Role of anti-CD14 antibody to protect α-MSH-mediated downregulation of CD14. Macrophages were incubated without or with 1 μg/ml anti-CD14 Ab for 2 h and then treated with different concentrations of α-MSH for 24 h. Cells, then washed with 0.5 M KSCN for 10 sec and then incubated with 1 μg/ml anti-CD14 Ab for 2 h followed by ^{125}I-IgG for 1 h at 37^{0}C. Then specific ^{125}I-IgG (mouse) binding was assayed as described in Materials and methods.

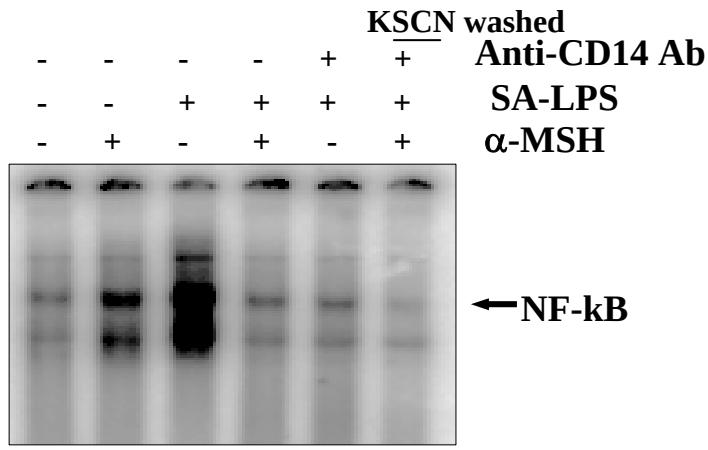


Fig. 4.6.4. Role of anti-CD14 antibody to protect α-MSH-mediated downregulation of NF-κB. Macrophages were incubated without or with 1 μg/ml anti-CD14 Ab for 2 h and then treated with different concentrations of α-MSH for 24 h. Cells, then washed with 0.5 M KSCN for 10 sec were incubated with 1 μg/ml anti-CD14 Ab for 2 h followed by ^{125}I-IgG for 1 h at 37^{0}C and specific binding was assayed. Cells, after KSCN washing were stimulated with SA-LPS (100 ng/ml) for 2 h, nuclear extract were prepared, and assayed for NF-κB by EMSA.

binding was then calculated. The result indicated in Figure 4.6.3 reveal that α-MSH decreased CD14 level (by decreasing ^{125}I-IgG binding) in a concentration dependent manner. Similar pattern of CD14 binding was shown in cells, pre-incubated with anti-CD14 followed by α-MSH treatment and washed with KSCN. From this result it is clear that anti-CD14 antibody does not protect α-MSH-mediated downregulation of CD14. Anti-CD14 Ab pre-incubated macrophages when treated with α-MSH showed inhibition of SA-LPS-induced NF-κB activation (Fig.4.6.4) further indicating the downregulation of CD14 by α-MSH and anti-CD14 Ab is unable to protect α-MSH mediated CD14 dowregulation.

4.3 Discussion

Research in the last two decades has proved the anti-inflammatory efficacy of α-MSH. α-MSH exerts its functions through its receptors (melanocortin receptor, MC-R) (Grantz *et al.,* 1993; Roselli-Rehfuss *et al.,* 1993) expressed on different cells of the body. It was reported that both human and murine macrophage cell lines express MC-R (Rajora N, 1996). Thus, it is likely that α-MSH can modulate their activities. Several inflammatory stimuli lead to activation of NF-κB followed by NF-κB-dependent gene expression. It is reported that α-MSH inhibits activation of NF-κB in monocytic cell line U-937 (Manna and Aggarwal, 1998). Macrophages play a central role during microbial infection, where in the inflammatory response is triggered by LPS, a major component of their outer membrane, via activation of NF-κB. Thus, the regulation of this transcription factor in macrophages appears to be clinically important. Upon infection, macrophages release several proteolytic enzymes to remove microbes *in vivo*. Stimulation of macrophages with SA-LPS led to release of myeloperoxidase and β-D-glucuronidase and α-MSH inhibited SA-LPS-induced release of those enzymes. However, there was no detectable change in alkaline phosphatase activity (Fig.4.1.1). Myeloperoxidase and β-D-glucuronidase are released from azurophilic granules but alkaline phosphatase is released from secretory vesicles (Manna and Samanta, 1995). SA-LPS might activate the macrophages by stimulating the release of azurophilic contents but not the secretory

vesicles. Activated cells show oxidative burst response and generate different oxygen species. Nitro blue tetrazolium (NBT) dye reacts with those oxygen species, converts into formazan granules and gets deposited inside the cells. α-MSH inhibited SA-LPS induced oxidative burst response as detected by NBT positive cells count (Fig.4.1.2). Oxidative burst response causes generation of different reactive oxygen and nitrogen species. α-MSH inhibited SA-LPS-induced nitrite and ROI generation (Fig.4.1.3).

In this report we observed that, endotoxin induced activation of NF-κB was inhibited by α-MSH (Fig.4.2.1). α-MSH also downregulated SA-LPS induced expression of ICAM-1 (Fig.4.2.4). This downregulation of NF-kB was further confirmed by NF-kB-dependent reporter gene (secretory alkaline phosphatase activity) assay (Fig.4.2.3). Several genes such as cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules which are involved in inflammation and tumor promotion, are regulated by NF-κB (Hwang *et al.*, 1997; Sato H and Seiki M, 1993; Collins *et al.*, 1995; Iademarco *et al.*, 1992). As α-MSH blocked activation of NF-κB and its dependent reporter gene expression, so it may play a critical role in inflammation.

α-MSH-mediated biological activities were observed not only in macrophages but also in human T-cell line and human neutrophils isolated from fresh human blood. However, why α-MSH did not show downregulation of NF-κB in human glioma cells is not understood. The level of α-MSH receptors on glioma cell surface had shown lesser than macrophages, neutrophils, or T-cells (Table-1). The low level of α-MSH receptor may be the cause of α-MSH in-sensitivity in glioma cells. α-MSH downregulated CD14, an endotoxin receptor, but not TNF receptors 1 and 2 (TNFR1 and TNFR2) or IL-1 receptors from macrophages cell surface (Fig.4.5). Recently Vega and Maio (2003) reported that geldanamycin downregulated CD14 from macrophages and it internalized and localized in cytoplasmic organelle, the endoplasmic reticulum (Vega, V.L, 2003). Our results suggest that α-MSH downregulates CD14 from macrophages by secreting it from cell surface but not by internalizing it in any cytoplasmic organelle. The possibility that, α-MSH might stimulate release of certain proteases from macrophages, which might be

responsible for the downregulation of CD14 and thereby inhibition of SA-LPS mediated biological activities, cannot be ruled out. Nevertheless, in this part of the work, we are providing a novel mechanism by which endotoxin mediated inflammatory responses are ameliorated by α-MSH indicating its anti-inflammatory activity in macrophage-driven inflammation. Thus, α-MSH may prove to be beneficial in treatment of sepsis.

CHAPTER FIVE

MODULATION OF IL-8 MEDIATED NEUTROPHIL FUNCTIONS BY α-MELANOCYTE STIMULATING HORMONE

5.1 Introduction

Chronic inflammatory diseases such as rheumatoid arthritis, gout, asthma, or inflammatory bowel disease are characterized with uncontrolled accumulation of neutrophils at the site of infection and residential macrophages liberate inflammatory molecules such as cytokines, reactive oxygen intermediates, and proteolytic enzymes which become major contributors to tissue damage (Pillinger and Abramson, 1995., Dallegri and Ottonello, 1997). Thus, regulation of recruitment of neutrophils and macrophages into inflammatory sites and their clearance are critical processes assuring effective host defense without tissue injury. An important mediator of these events is interleukin-8 (IL-8) (Baggiolini *et al.,* 1994., Baggiolini *et al.,* 1997**)**, a potent neutrophil chemotactic agent (Oppenheim *et al.,* 1991**)**. It triggers respiratory burst response, degranulation, and stimulates neutrophil adhesion to the endothelial cells (Baggiolini *et al.,* 1995., Samanta *et al.,* 1989., Besemer *et al.,* 1989 **)**. IL-8 interacts with its receptor, IL-8R also known as CXCR. The IL-8Rs are of two types (type A and B). The type A receptor (IL-8R1 or CXCR1) binds IL-8 with high affinity but shows low affinity to melanocyte growth stimulatory activity (MGSA) whereas type B receptor (IL-8R2 or CXCR2) binds to IL-8 and MGSA with high affinity (Jones *et al.,* 1996**)**. The possible strategies for ameliorating the inflammatory distress may be either by prevention of excessive neutrophil migration by reducing the interaction of neutrophils with the inflammatory cytokine or by regulating IL-8 production.

Receptors of α-MSH have been detected on both monocyte/macrophages and neutrophils (Star *et al.,* 1995., Watanabe *et al.,* 1993**)**. In continuation with our quest to understand how α-MSH regulates inflammation induced by different stimuli and in different cells, we extended our study to yet another very important player in inflammation, the neutrophils. In this study, we were trying to understand the anti-inflammatory effect of α-MSH on neutrophils and macrophages, isolated from human blood. With the already established role of IL-8 in neutrophil recruitment, we wanted to study the regulation of neutrophil function by α-MSH in presence of IL-8.

5.2 Results

In this study, we examined the effect of α-MSH on IL-8-induced biological responses in macrophages and neutrophils. DMSO was used to convert HL-60 cells to neutrophils in 2 days and THP1 cells were converted into macrophages by induction with PMA for 16 h. These cells were used for most of the experiments. After conversion, neutrophils were cultured up to 48 h without significant decrease in cell death (cell viability was 99.02 ± 2.24, 97.34 ± 3.36, 94.78 ± 3.24, and 85.46 ± 5.52 % at 0, 24, 36, and 48 h of incubation respectively).

α-MSH inhibits IL-8-induced NF-κB activation.

To detect the role of α-MSH on IL-8-induced NF-κB activation in neutrophils and macrophages, cells were incubated with different concentrations of α-MSH for 24 h and then stimulated with IL-8 (100 ng/ml) for 2 h at 37^0C. Nuclear extract (NE) was prepared and assayed for NF-κB by gel shift assay as described in Materials and Methods. IL-8 induced NF-κB in neutrophils (Fig.5.1.1A) and macrophages (Fig.5.1.1B). α-MSH alone did not activate NF-κB, but inhibited IL-8-induced NF-κB activation in a dose dependent manner. At 100 nM, α-MSH completely abrogated IL-8-induced NF-κB activation in both cells. To rule out the possibility of endotoxin contamination in IL-8 induced NF-κB, cells were stimulated with either IL-8 or IL-8-polymixin B sulphate mixture. NE was then assayed for NF-κB. The results as shown in Figure 5.1.1C indicate that, polymixin B sulphate pre-incubated IL-8 activated NF-κB similar to IL-8, suggesting the IL-8-mediated activation of NF-κB is not due to endotoxin.

α-MSH inhibits IL-8-induced myeloperoxidase, alkaline phosphatase, or β-D glucuronidase activities.

To detect the α-MSH-mediated effect on IL-8-induced proteolytic enzymes release, untreated and α-MSH-treated neutrophils were stimulated with different concentrations of IL-8 for 2 h and culture supernatant was assayed for

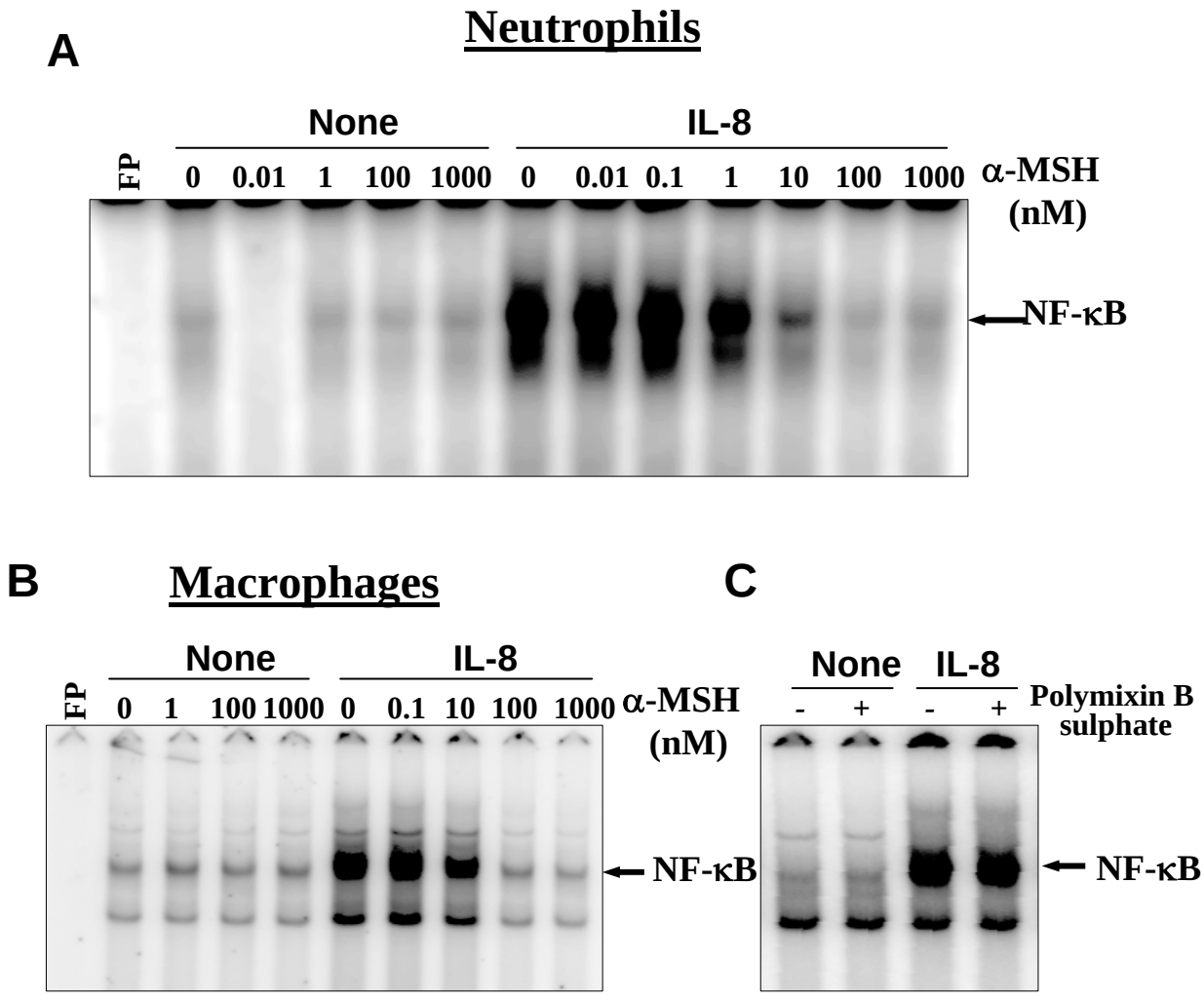


Fig.5.1.1. Effect of α-MSH on IL-8-induced NF-κB activation. HL-60 cells differentiated neutrophils (**A**) and THP1 cells differentiated macrophages (**B**) were incubated with different concentrations of α-MSH for 24 h at 37^0C, CO_2 incubator. Then cells were stimulated with 100 ng/ml IL-8 for 2 h. Nuclear extracts were prepared and 8 μg NE proteins were assayed for NF-κB. 100 ng IL-8 was incubated with 10 μg of polymixin B sulphate for 1 h at 37°C. Neutrophils were stimulated with this mixture or IL-8 (100 ng/ml) for 2 h. Nuclear extracts were prepared and assayed for NF-κB (**C**).

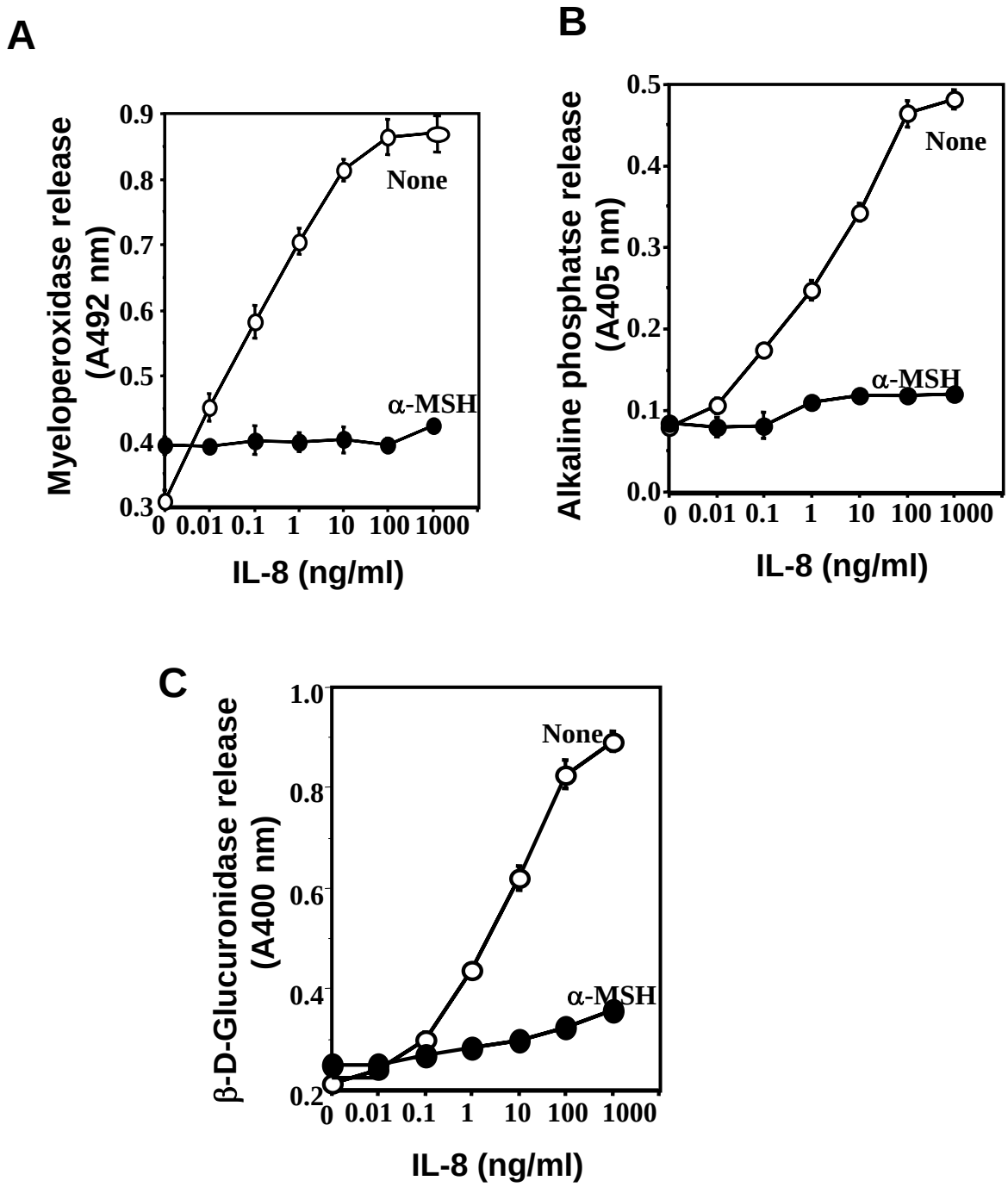


Fig.5.2. Effect of α-MSH on IL-8-induced enzymes release. Neutrophils, treated with 1000 nM α-MSH for 24 h were stimulated with different concentrations of IL-8 for 2 h in triplicate samples. The supernatant was collected and analyzed for myeloperoxidase (**A**), alkaline phosphatase (**B**), and β -D-glucuronidase (**C**).

myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase (Fig.5. 2 A, B, C, respectively). The absorbencies for all three enzymes were enhanced with increasing concentrations of IL-8, which was inhibited by α-MSH pre-treated cells. α-MSH alone showed marginal increase in myeloperoxidase level in neutrophils. From these results it is clear that IL-8-mediated release of proteolytic enzymes is inhibited by α-MSH.

α-MSH inhibits IL-8-, but not FMLP-induced neutrophil migration.

IL-8- or FMLP-, induced neutrophil migration (as shown by chemotactic index, determined from induced migrated cell numbers divided by un-induced migrated cell numbers) in a time-dependent manner. α-MSH-pre-treated cells showed 60-70% decrease in migrated cells number at any time of incubation with IL-8, but not with FMLP (Fig. 5.3.1A & 5.3.1B). This result suggests that α-MSH inhibits IL-8-, but not FMLP-induced neutrophil migration, which correlates with downregulation of activation of NF-κB and release of proteolytic enzymes.

α-MSH inhibits IL-8-, but not FMLP induced oxidative burst response.

IL-8- or formyl peptide (FMLP)-induced generation of reactive oxygen species (ROS) in neutrophils is well established (Manna *et al.,* 1997). The generation of ROS is detected by NBT (nitro blue tetrazolium) test. Does α-MSH modulate only IL-8-mediated biological responses? Are other chemoattractant-mediated biological responses also affected? To address these questions, neutrophils, treated with different concentrations of α-MSH for 24 h were stimulated with IL-8 or FMLP for 2 h at 37^0C in presence of NBT solution (0.1%). NBT positive cells were counted under microscope and presented in percentage above unstimulated cells (Fig.5.3.2). α-MSH did not increase the number of NBT positive cells alone in unstimulated cells. IL-8 and FMLP induced 66 and 80% increase in NBT positive cells respectively. However, α-MSH inhibited IL-8-, but not FMLP-induced NBT positive cells indicating its specificity towards IL-8.

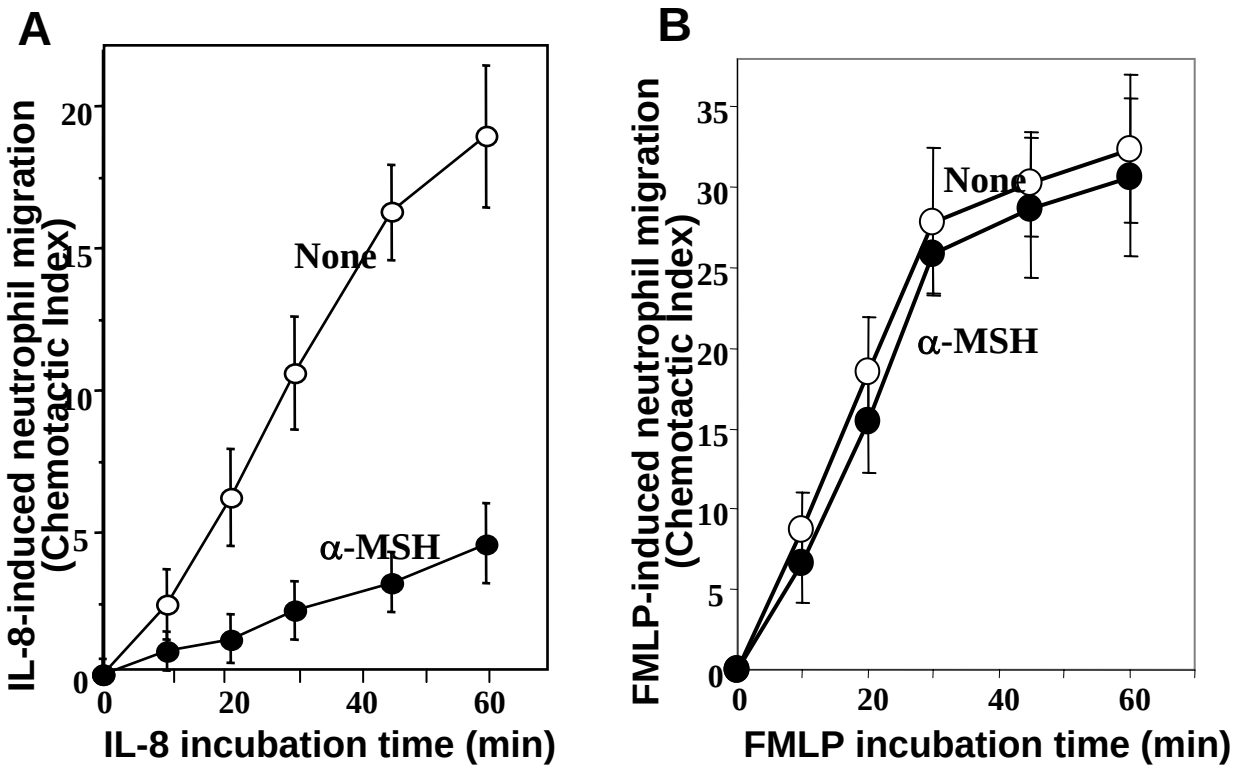


Fig. 5.3.1. Effect of α-MSH on IL-8- or FMLP-induced neutrophil migration. Neutrophils were treated with α-MSH (1000 nM) for 24 h and then IL-8 (100 ng/ml)- or FMLP (100 nM)-induced migration in Boyden chemotactic chamber for different times. The migrated cells were stained with Giemsa and counted under microscope. Chemotactic index was calculated from IL-8 (**A**)- or FMLP (**B**)-induced divided by non-induced migrated cells of three independent experiments.

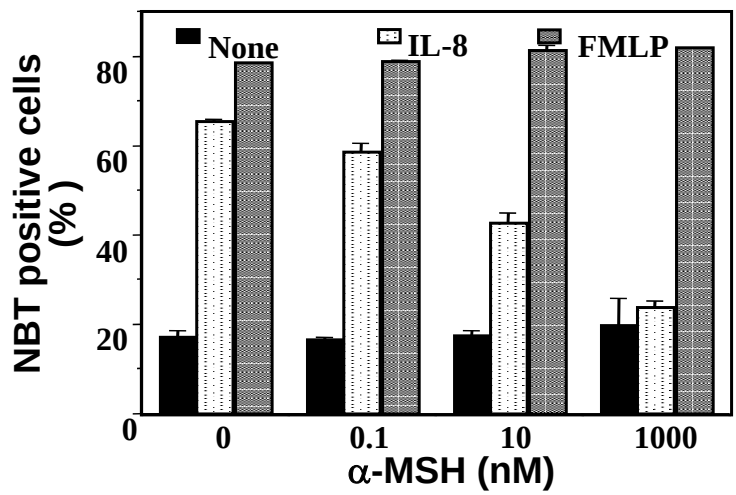


Fig.5.3.2. Effect of α-MSH on IL-8- or FMLP-induced oxidative burst response. Neutrophils, treated with different concentrations of α-MSH for 24 h, were stimulated with 100 ng/ml IL-8 or 100 pM FMLP for 2 h. Then NBT test was carried out. NBT positive and negative cells were counted under microscope and NBT positive cells were presented in percentage.

α-MSH is unable to protect FMLP-induced NF-κB activation.

As α-MSH was unable to inhibit FMLP-induced neutrophils migration and oxidative burst response, the FMLP-induced NF-κB activation was also assayed. α-MSH treated cells did not block FMLP-induced NF-κB activation at any concentration (Fig.5.3.3), further suggesting α-MSH's specific action on IL-8-, but not FMLP-induced biological responses.

α-MSH inhibits IL-8-, but not FMLP-induced NF-κB-dependent SEAP activation.

To further prove α-MSH-mediated inhibition of NF-κB activation, HL-60 cells, transfected with NF-κB responsive reporter plasmid containing SEAP gene followed by differentiation into neutrophils as described before, were treated with α-MSH, stimulated with IL-8 or FMLP for 12 h and the culture supernatant was assayed for secretory alkaline phosphatase (SEAP) activity. The result indicated that IL-8 (Fig.5.3.4A) or FMLP (Fig.5.3.4B) induced SEAP activity in a dose-dependent manner. However, IL-8-, but not FMLP-induced SEAP activity was completely blocked in α-MSH-pretreated cells. The results suggest that α-MSH inhibits IL-8-induced NF-κB-dependent gene expression.

α-MSH inhibits IL-8, but not TNF, TRAIL, IL-4, or IL-13 binding.

As α-MSH inhibited IL-8-induced biological responses and IL-8 exerts its effect through surface CXCRs, the effect of α-MSH on expression of different receptors on neutrophils were detected. Neutrophils (1×10^6/2 ml), treated with different concentrations of α-MSH for 24 h in triplicate samples, were incubated with ^{125}I-labeled TNF, TRAIL, IL-4, IL-13, or IL-8 (5×10^4 cpm/tube) and binding was detected. The results indicated in Figure 5.4.1 reveals that ^{125}I-IL-8 binding was decreased with increasing concentrations of α-MSH (79% at 1000 nM α-MSH) but not for the binding of TRAIL, IL-4, or IL-13. A marginal decrease in TNF binding (16% at 1000 nM of α-MSH) was observed. The results indicate that α-MSH inhibits

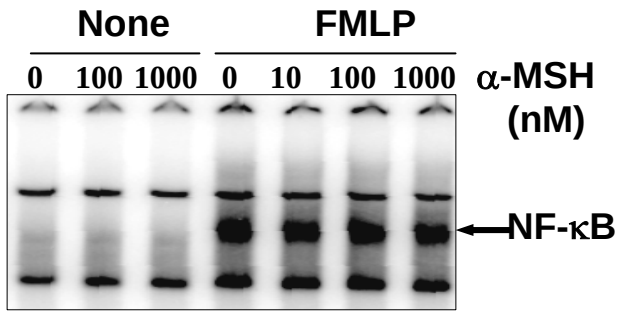


Fig. 5.3.3. Effect of α-MSH on FMLP-induced NF-κB activation. Neutrophils, treated with different concentrations of α-MSH for 24 h were stimulated with 100 pM of FMLP for 2 h. Nuclear extracts were assayed for NF-κB by gel shift assay.

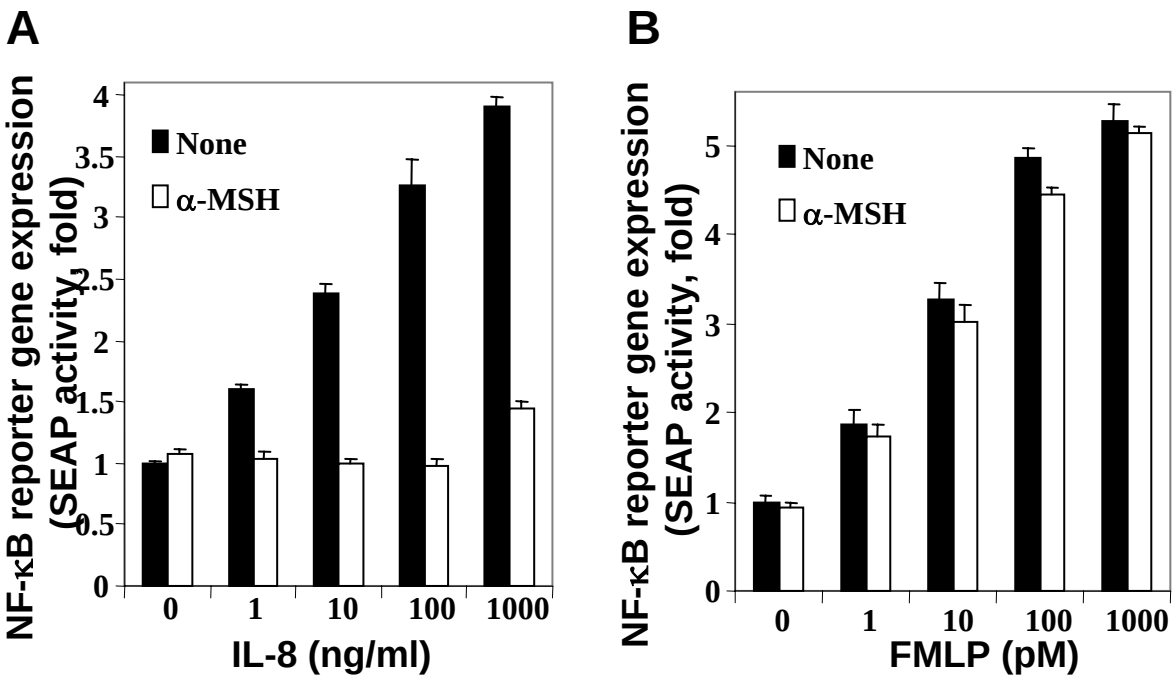


Fig.5.3.4. Effect of α-MSH on IL-8- or FMLP-induced NF-κB-dependent reporter gene expression. HL-60, transiently transfected with indicated plasmids along with NF-κB-containing plasmid linked to the SEAP gene, were cultured in presence of 1.3% DMSO for 2 days. Cells, untreated or pre-treated with α-MSH for 24 h were stimulated with different concentrations of IL-8 (**A**) or FMLP (**B**) for another 12 h. Then cultured supernatant was taken and assayed for SEAP.

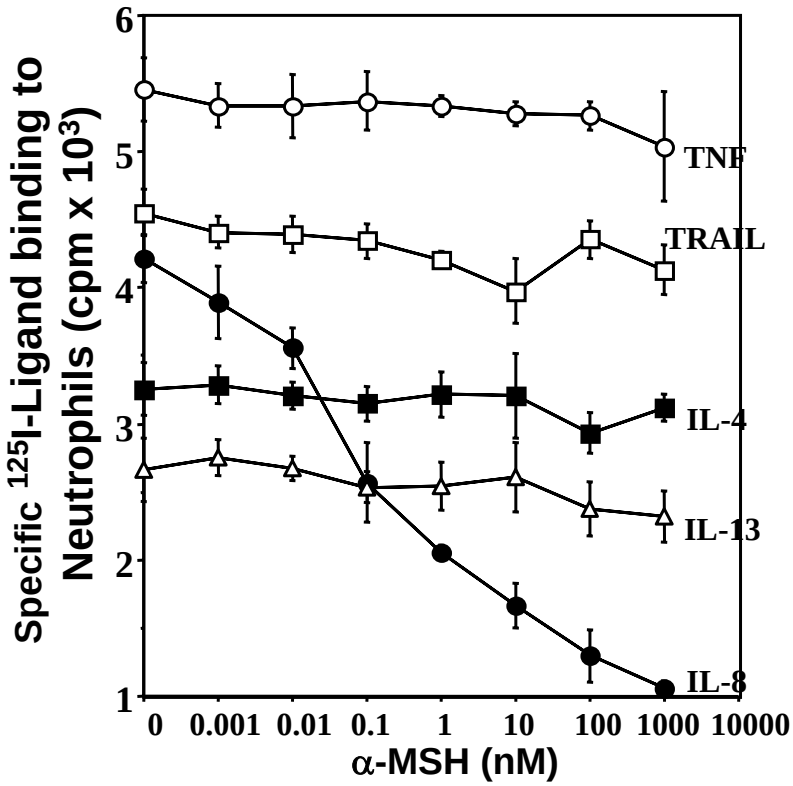


Fig. 5.4.1. Effect of α-MSH on IL-8, TNF, TRAIL, IL-4, or IL-13 binding. Neutrophils (1 x 10^6/2 ml) were incubated with different concentrations (0-1000 nM) of a-MSH for 24 h at 37^0C, CO_2 incubator. Labeled TNF, TRAIL, IL-4, IL-13, and IL-8 binding was assayed at 4^0C.

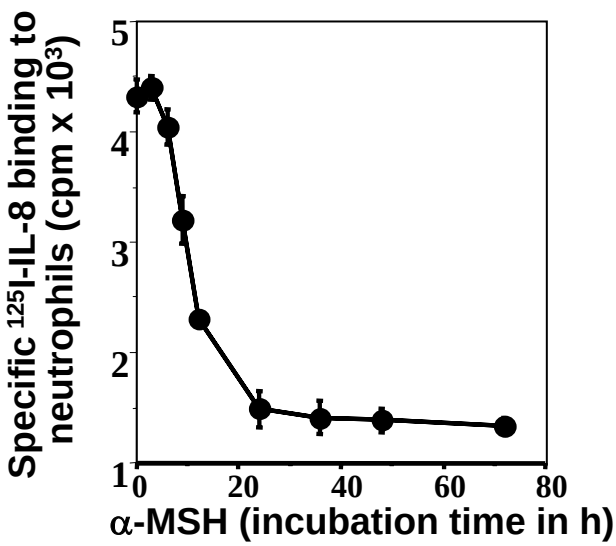


Fig.5.4.2. Detection of optimum time of α-MSH treatment to inhibit IL-8 binding. Neutrophils (1 x 10^6/well of 12 well plate) were incubated with 1000 nM of α-MSH for different times as indicated and ^{125}I-IL-8 binding was assayed of triplicate samples.

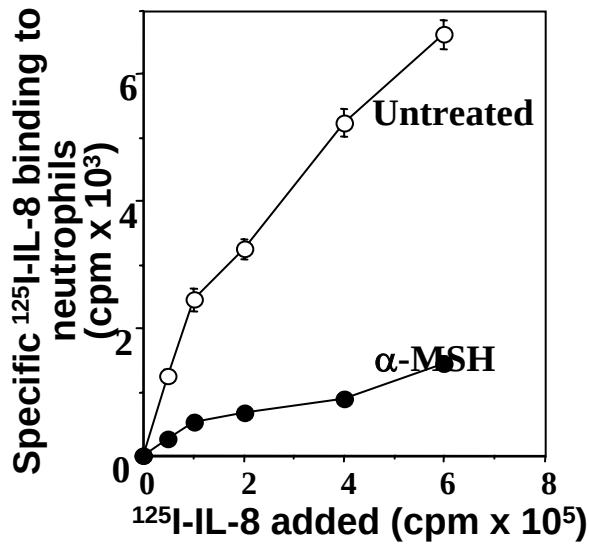


Fig.5.4.3. Effect of α-MSH on ^{125}I-IL-8 binding. Neutrophils were treated with 1000 nM α-MSH for 24 h at 37^0C and then incubated with different amount of ^{125}I-IL-8 at 37^0C for 1 h.

binding of IL-8, but not TNF, TRAIL, IL-4, or IL-13 in neutrophils in a dose-dependent manner. To detect the optimum time of α-MSH treatment to inhibit maximum IL-8 binding, neutrophils were treated with 1000 nM α-MSH for different times and ^{125}I-IL-8 binding was assayed at 4^0C. As shown in Figure 5.4.2, the IL-8 binding was gradually decreased up to 24 h. From this result it is clear that, optimum time required for maximum inhibition of IL-8 binding by α-MSH is 24 h. Viable receptors were detected by accumulation of labeled ligand inside the cells (total binding). As shown in Figure 5.4.3, the accumulated ^{125}I-IL-8 was decreased in α-MSH-treated cells to about 70-80% at any concentrations of added ^{125}I-IL-8 compared to untreated cells further suggesting downregulation of CXCRs by α-MSH.

α-MSH interacts predominantly with MC-1 receptors in neutrophils and macrophages.

To detect the type of melanocortin receptors used by α-MSH in neutrophils and macrophages to downregulate CXCRs, different cells (THP-1, HL-60, differentiated neutrophils and macrophages, isolated neutrophils and macrophages from blood, and melanoma cells-A375) were cultured in complete medium for 24 h at 37^0C and extracts were analyzed for MC-1R, MC-2R, MC-3R, or MC-4R by Western blot. The level of MC-1R was increased in differentiated and isolated neutrophils and macrophages. All those MC-Rs were expressed in melanoma cells significantly (Fig.5.5.1). Further to detect the type of MC-Rs used by α-MSH to downregulate CXCRs, cells were pre-incubated with 1 μg of each of MC-1R, MC-2R, MC-3R, MC-4R, or all for 1 h, treated with α-MSH for 24 h and IL-8 binding was assayed. α-MSH inhibited 79% of IL-8 binding. Cells, pre-incubated with Anti-MC-1R, -MC-2R, -MC-3R, -MC-4R, or all these antibodies, followed by α-MSH-treatment, showed 25, 70, 67, 78, or 10% inhibition of IL-8 binding respectively (Fig.5.5.2) suggesting that α-MSH exerts its signal mostly through MC-1R in neutrophils.

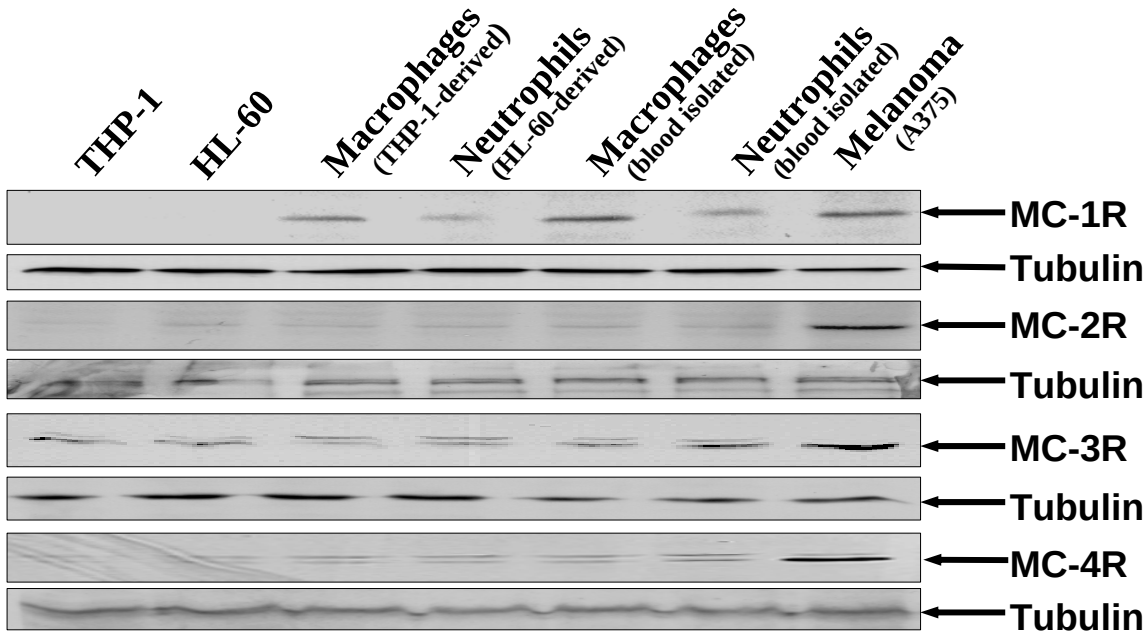


Fig.5.5.1. Detection of different melanocortin receptors in neutrophils and macrophages. THP-1, HL-60, differentiated macrophages and neutrophils, human blood isolated neutrophils and macrophages, and melanoma (A375) cells, cultured for 24 h at 37^0C in complete medium were extracted and 200 μg extract proteins were used to detect MC-1R, MC-2R, MC-3R, and MC-4R by Western blot. Blots were reprobed with anti-tubulin antibody to detect tubulin.

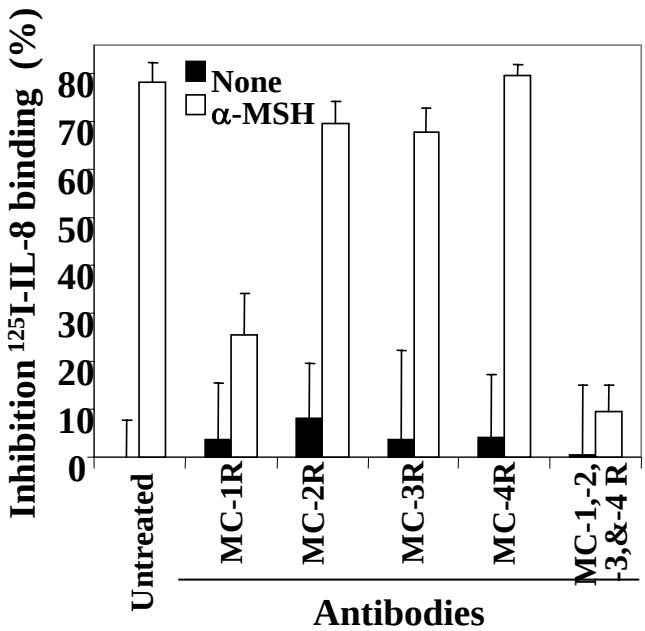


Fig.5.5.2. Effect of different anti-MC receptors antibodies on α-MSH-mediated inhibition of IL-8 binding. Neutrophils, pre-incubated with 1 μg of each of anti-MC-1R, -MC-2R, -MC-3R, or –MC-4R antibody or all these antibodies for 1 h were incubated with α-MSH for 24 h at 37^0C in triplicate. Cells were washed and ^{125}I-IL-8 binding was assayed.

α-MSH does not compete with IL-8 binding.

To detect the effect of α-MSH on IL-8 binding site in neutrophils, cells were pre-incubated with 250 ng of unlabeled IL-8 or α-MSH for 4 h at 4^0C in triplicate followed by incubation with different amounts of ^{125}I-IL-8 for 2 h and binding was assayed. Unlabeled IL-8, but not α-MSH almost completely suppressed labeled IL-8 binding (Fig.5.5.3) suggesting α-MSH does not compete with IL-8 binding site in neutrophils.

α-MSH inhibits IL-8 binding.

To estimate the number of CXCRs expressed in α-MSH treated neutrophils, Scatchard analysis was performed (Manna *et al.,* 1997., Manna and Aggarwal 1998). Cells, either untreated or treated with α-MSH (1000 nM) for 24 h were incubated with different concentrations of ^{125}I-IL-8 at 4^0C for 2 h in presence or absence of 50 fold unlabeled IL-8. The specific binding of IL-8 was shown in Figure 5.6.1 as mean count ± SD of duplicate samples. From specific count, bound/free values were calculated and plotted against bound values (Fig.5.6.1 A2, inset). From these data total number of CXCR was calculated as 16,958 CXCRs per unstimulated neutrophil (Kd, 0.5 nM) versus 3,390 (Kd, 0.4 nM) by α-MSH treated neutrophils. α-MSH downregulates about 79% CXCRs as detected by Scatchard plot that is correlated with IL-8 binding data without changing affinity towards IL-8.

To demonstrate α-MSH-mediated downregulation of IL-8 binding, chemical coupling of ^{125}I-IL-8 to receptors was performed at 4^0C by a bifunctional cross-linker DSS. Figure 5.6.2A showed that the intensity of 67 and 75 kDa bands were decreased with increasing concentrations of α-MSH, thus indicating downregulation of CXCRs. To detect the type of CXCRs downregulated by α-MSH, cells were treated with α-MSH (1000 nM) for 24 h and then incubated with 250 ng of IL-8 or MGSA for 2 h at 4^0C and then ^{125}I-IL-8 binding was assayed. The 50-fold unlabeled IL-8 suppressed almost 90% labeled IL-8 binding, whereas cold MGSA suppressed about 50% labeled

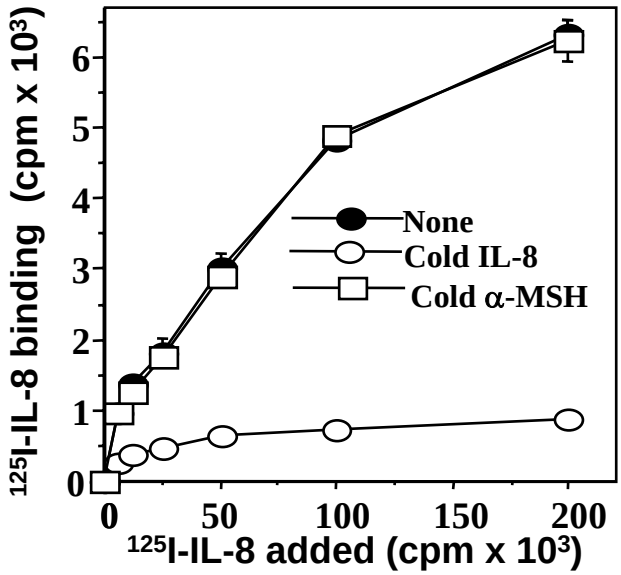


Fig.5.5.3. Effect of cold α-MSH in IL-8 binding. Neutrophils (2 x 10^6 cells/200 μl) were incubated without or with IL-8 (250 ng) or α-MSH (250 ng) at 4^0C for 4 h and then incubated with different concentrations of labeled IL-8 for 2 h at 4^0C.

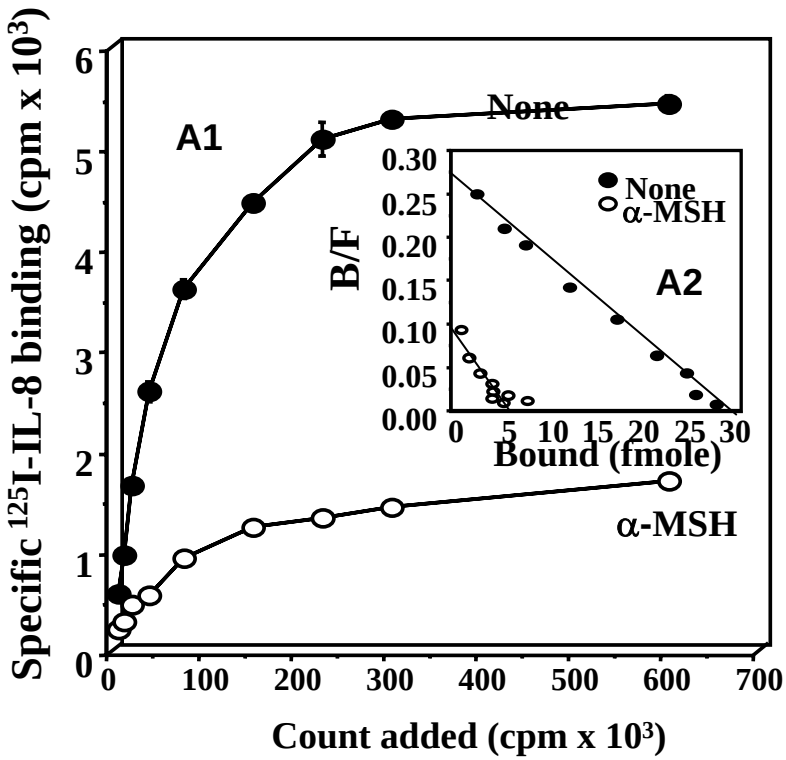


Fig. 5.6.1. Scatchard analysis of ^{125}I-IL-8 binding to α-MSH-treated neutrophils. α-MSH-treated and untreated neutrophils (1 x 10^6) were incubated without or with 200 ng of IL-8 in duplicate. Then IL-8 binding was assayed using different amounts of ^{125}I-IL-8 at 4^0C for 2 h. To determine the specific binding, nonspecific binding (obtained from a 50-fold excess of cold IL-8 used in binding) was subtracted (**A1**). The result shown is representative of three independent experiments. From specific binding, ligand bound versus bound/free ratio was indicated (**A2**).

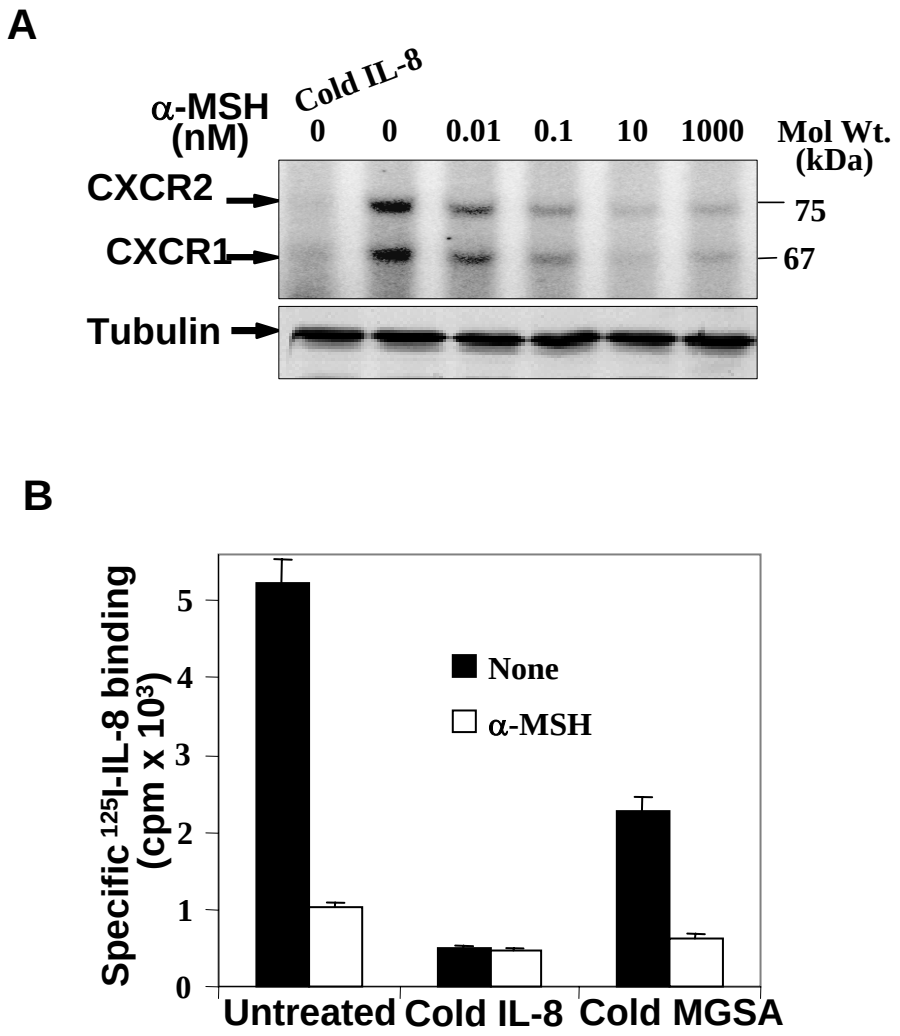


Fig.5.6.2. A. Effect of α-MSH on IL-8 binding. Neutrophils were treated with different concentrations of α-MSH for 24 h and then ^{125}I-IL-8 binding was done. Neutrophils were used to cross-link the ligand–receptor using DSS, cell extract was prepared and 250 μg protein was analyzed in 10% SDS-PAGE and the dried gel was scanned in PhosphorImager. 50 μg proteins was probed for tubulin by Western blot for loading control. **B**. **Detection of specific IL-8 binding to neutrophils.** Neutrophils were treated with 1000 nM α-MSH , washed, incubated with 250 ng of unlabeled IL-8 or MGSA for 30 min followed by labeled IL-8 for 2 h at 4^0C and binding was assayed. Results represented one out of three experiments.

IL-8 binding (Fig.5.6.2B) suggesting the downregulation of both types of CXCRs by α-MSH.

Downregulation of CXCRs was also detected by immunofluorescence. Cells, after α-MSH treatment, were fixed, incubated with anti-CXCR1 and anti-CXCR2 antibodies, followed by staining with anti-rabbit IgG-Alexa-Fluor and DAPI. The cells were visualized under immunofluorescence microscope. The Alexa-Fluor level was significantly decreased in α-MSH-treated cells (Fig.5.6.3A) indicating the downregulation of CXCRs. α-MSH-mediated downregulation of CXCR1 was also detected from cells extract immunoprecipitated with anti-CXCR1 antibody followed by Western blot with anti-CXCR1 antibody (Fig.5.6.3 B).

α-MSH does not inhibit CXCRs mRNA.

As α-MSH downregulated CXCRs, the levels of CXCR1 and CXCR2 mRNA were determined by RT-PCR. The total RNA was isolated from neutrophils, treated with α-MSH (1000 nM) or LPS (100 ng/ml) for 24 h. This RNA was used for RT-PCR followed by PCR using CXCR1, CXCR2, or actin specific primers and analyzed in 1% agarose gel. The bands for CXCR1or CXCR2 did not change in α-MSH-treated cells. LPS induced these bands and actinomycin D inhibited LPS-induced CXCR1 and CXCR2 (Fig.5.6.4) suggesting that α-MSH-mediated downregulation of CXCRs is not at the mRNA level.

α-MSH does not inhibit IL-8 binding in isolated neutrophil membrane or purified CXCRs.

To detect the effect of α-MSH in neutrophils membrane and purified CXCRs, neutrophils, isolated membrane, and affinity purified CXCRs were incubated with different concentrations of α-MSH for 24 h at 37^0C. Labeled IL-8 binding was assayed. The results shown in Figure 5.7.1 A indicate that ^{125}I-IL-8 binding was decreased in neutrophils, but not in neutrophil membrane or purified CXCRs. From

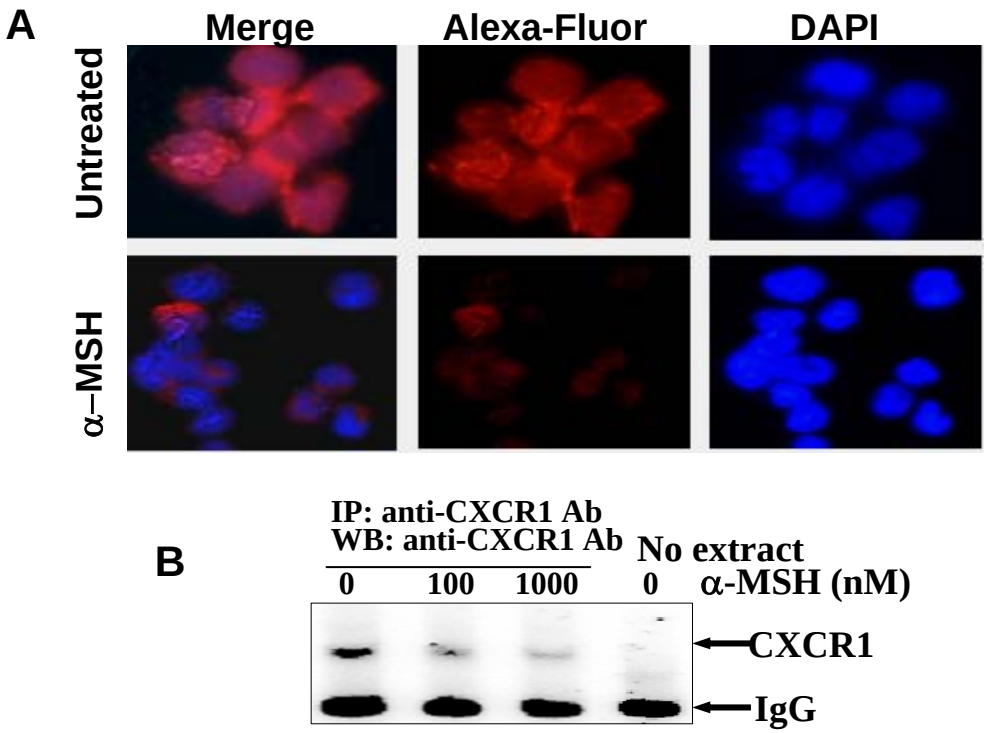


Fig. 5.6.3. Effect of α-MSH on CXCRs levels. A. Neutrophils, treated with α-MSH (1000 nM) for 24 h were fixed with 4% paraformaldehyde, incubated with anti-CXCR1 and –CXCR2 antibodies followed by Alexa-Fluor conjugated anti-rabbit IgG. Cells were mounted with mounting medium containing DAPI and visualized in fluorescence microscope. **B.** Neutrophils, treated with different concentrations of α-MSH for 24 h were extracted and 300 μg extract proteins were immunoprecipitated with anti-CXCR1 antibody and detected for CXCR1 by Western blot.

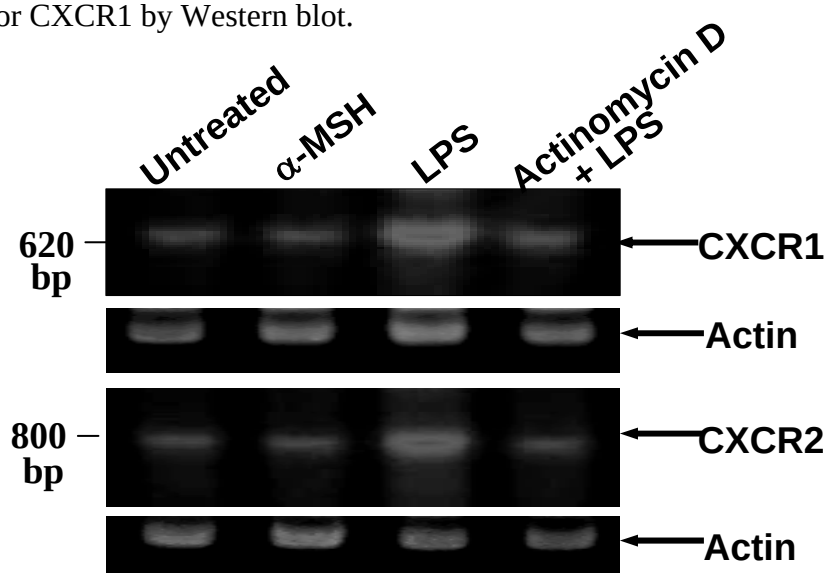


Fig. 5.6.4. Effect of α-MSH on CXCRs mRNA level. Neutrophils were treated with α-MSH (1000 nM), LPS (100 ng/ml), or actinomycin D (1 mM) pretreated for 30 min followed by LPS for 24 h and then RNA was isolated. The isolated RNA was used to detect CXCR1, CXCR2, and actin using specific primers by RT-PCR and the products were detected in agarose gel.

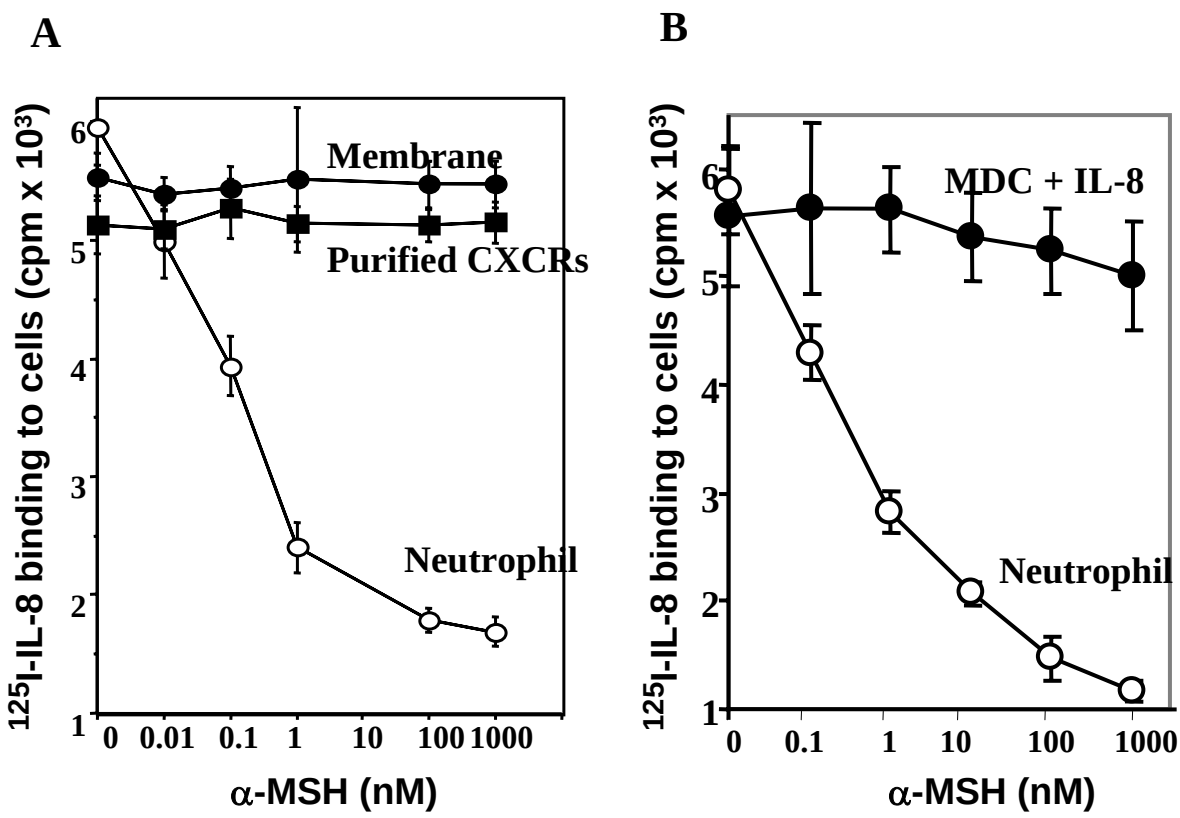


Fig. 5.7.1 A. Effect of α-MSH on purified neutrophils membrane and affinity purified CXCRs. Purified membrane and affinity purified CXCRs, taken on nitrocellulose discs and neutrophils were treated with different concentrations of α-MSH. The nitrocellulose discs and neutrophils were washed and then assayed for ^{125}I-IL-8 binding. **B. IL-8 protects α-MSH-mediated inhibition of IL-8 binding**. Neutrophils, pre-incubated with MDC for 15 min at 37^0C were incubated with IL-8 (500 ng/ml) for 1 h at 4^0C. After washing, the cells were treated with different concentrations of α-MSH for 24 h at 37^0C. Cells were washed with 0.05 M glycine-HCl, pH 3.0 and then assayed for ^{125}I-IL-8 binding.

these results, it is clear that α-MSH only interacts with cells, but not in isolated membrane or affinity purified CXCRs.

IL-8 or anti-CXCRs Abs protect α-MSH-mediated inhibition of IL-8 binding.

Whether α-MSH mediates CXCRs modulation within IL-8 binding domain of the receptor, the ligand protection experiment was performed. As monodansyl cadaverine (MDC) can protect endocytosis at 37^0C (Ray and Samanta, 1997), MDC-pretreated cells were incubated with 500 ng/ml IL-8 for 1 h at 4^0C. The cells were washed, treated with different concentrations of α-MSH for 24 h, and then washed with glycine-HCl (50 mM, pH 3). The binding of ^{125}I-IL-8 was assayed at 4^0C immediately after washing the cells. MDC treated cells did not show any alteration of IL-8 binding. IL-8 binding was decreased in neutrophils by α-MSH treatment in a dose-dependent manner, but IL-8 pre-incubated cells showed no inhibition of IL-8 binding (Fig.5.7.1B) indicating that α-MSH-mediated downregulation of CXCRs is protected by IL-8. To examine the protection of CXCRs from the effect of α-MSH by anti-CXCRs antibodies, cells, incubated with 1 μg of each anti-CXCR1 and –CXCR2 antibodies/2 x10^6 cells for 1 h at 37^0C were incubated with different concentrations of α-MSH at 37^0C, CO_2 incubator for 24 h. Cells were then washed with 0.5 M potassium thiocyanate (KSCN) for 10 sec. After immediate washing, the mean binding of ^{125}I-IL-8 (cpm) was assayed at 4^0C. The result indicated in Figure 5.7.2A suggests that α-MSH decreased IL-8 binding in a dose-dependent manner but anti-CXCRs Abs pre-incubated cells did not show α-MSH-mediated decrease in IL-8 binding, indicating protection of CXCRs by its antibodies.

α-MSH does not induce IL-8 production.

The relevant question is whether α-MSH-mediated CXCRs downregulation occurred simply by production of IL-8. To address this question, cells were treated with α-MSH and level of IL-8 was detected. Neutrophils were treated with 100 and 1000 nM of α-MSH or 100 ng/ml LPS for 24 h. Cell supernatant (10 times

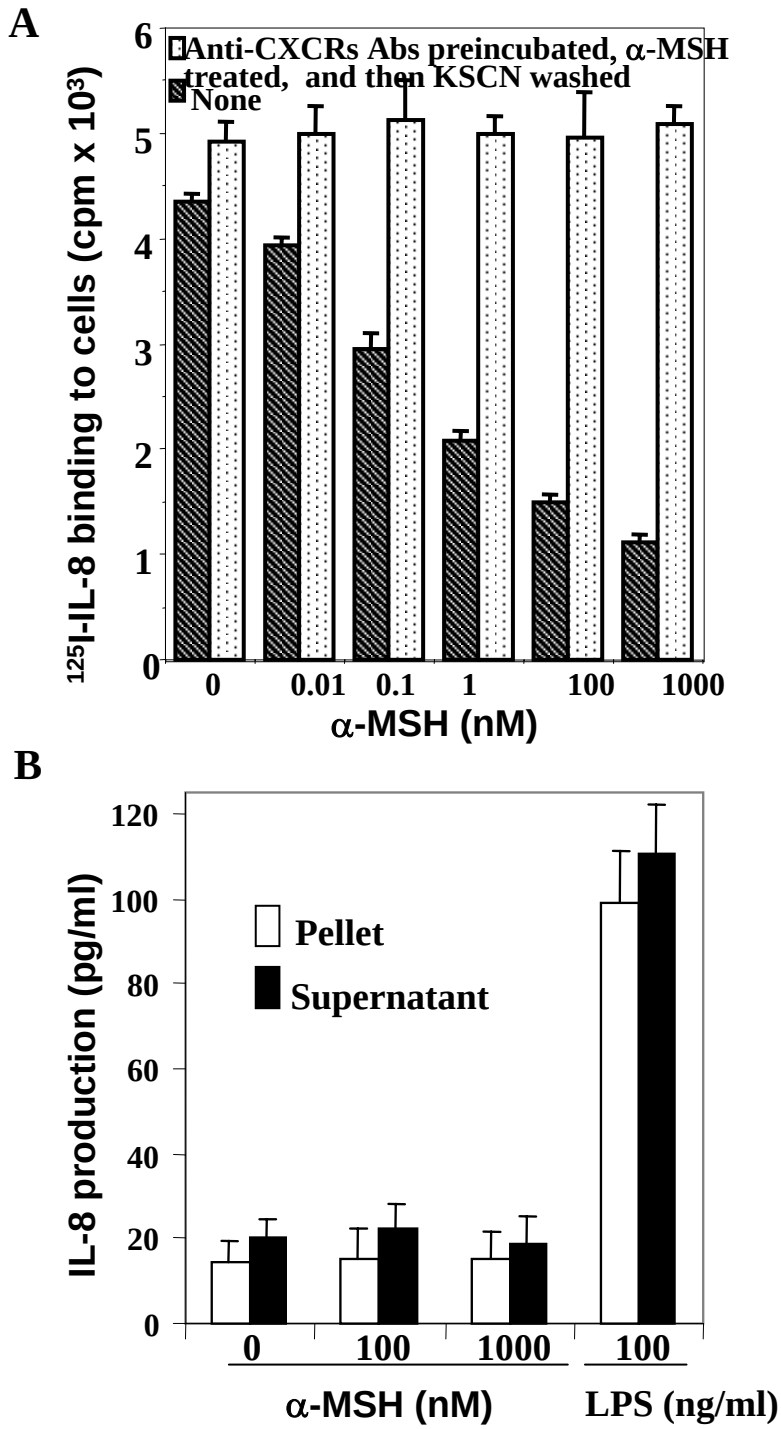


Fig. 5.7.2 A. Anti-CXCR1 and –CXCR2 Abs protect α-MSH-mediated inhibition of IL-8 binding. Cells, incubated with anti-CXCR1 and –CXCR2 Abs for 1 h, were treated with different concentrations of α-MSH for 24 h. Cells were washed with 0.5 M KSCN and assayed for ^{125}I-IL-8 binding. Results indicated is representative of three independent experiments. **B**. **α-MSH does not induce IL-8 production.** Neutrophils were treated with 100 and 1000 nM α-MSH or 100 ng/ml LPS for 24 h. Culture supernatant and cells extracts assayed for IL-8 level by ELISA using IL-8 assay kit. Results represented in pg/2x10^5 cells of IL-8.

concentrated) and extract (from pellet) were assayed for IL-8 using IL-8 assay kit. LPS induced significant increase in the level of IL-8 as detected from cell supernatant and pellet, whereas α-MSH-treated cells did not enhance the level of IL-8 expression (Fig.5.7.2B). The results suggest that α-MSH-mediated downregulation of CXCRs was not due to production of IL-8.

PMSF and CMK protect α-MSH-mediated inhibition of IL-8 binding.

As proteases are known to cleave different proteins from cell surface, it is important to detect the role of proteases in α-MSH-mediated downregulation of CXCRs. Neutrophils (1×10^6) were pre-incubated with different protease inhibitors for 2 h and subsequently treated with α-MSH (1000 nM) for 24 h. Then ^{125}I-IL-8 binding was assayed. The results indicated in Figure 5.8.1 show that the binding of IL-8 was not decreased by different protease inhibitors alone in neutrophils but pretreatment with PMSF or CMK (neutrophil elastase inhibitor) showed 80-90% protection and leupeptin, TPCK, or TLCK showed 40% protection of IL-8 binding in α-MSH- treated cells. The results indicate that leupeptin, TPCK, or TLCK partially, but PMSF or CMK almost completely protect α-MSH-mediated downregulation of CXCRs. The CMK protected α-MSH-mediated CXCRs downregulation in a dose-dependent manner (Fig. 5.8.2A). To establish the PMSF- or CMK-mediated protection of CXCRs, downregulated by α-MSH, IL-8 mediated NF-κB activation was assayed. Neutrophils were treated with α-MSH, pre-, co- or post- incubated with PMSF (100 μM) or CMK (1 μM). Then cells were induced with IL-8 for 2 h and NF-κB was assayed from nuclear extract. IL-8 induced NF-κB, which was downregulated by α-MSH. PMSF (Fig.5.8.2B) or CMK (Fig.5.8.2C) did not interfere with IL-8-induced NF-κB activation alone but pre-incubation of CMK or PMSF protected α-MSH-mediated downregulation of NF-κB but not by co- or post-incubation with α-MSH. The results clearly indicate that neutrophil elastase inhibitor blocks α-MSH-mediated downregulation of CXCRs and thereby NF-κB activation by IL-8.

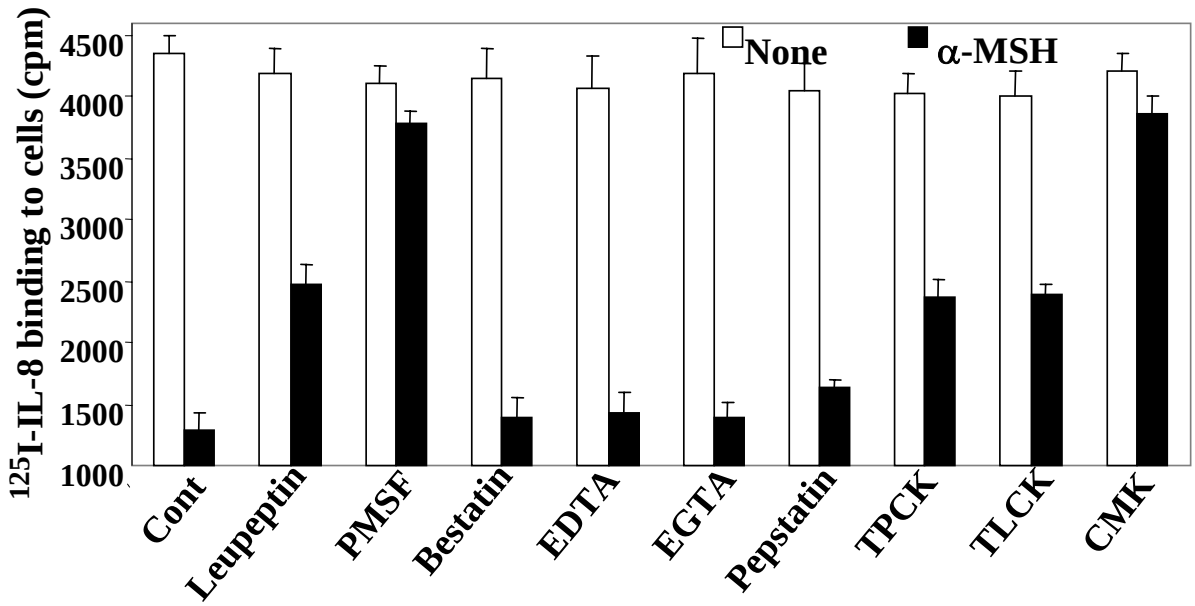


Fig. 5.8.1 Effect of protease inhibitors on α-MSH-mediated inhibition of IL-8 binding. Neutrophils, treated with 100 μM of leupeptin, PMSF, bestatin, EDTA, EGTA, pepstatin, TPCK, TLCK, or TPCK and 1 μM CMK for 2 h were treated with α-MSH (1000 nM) for 24 h at 37^0C. Then ^{125}I-IL-8 binding was carried out as described in Materials and Methods.

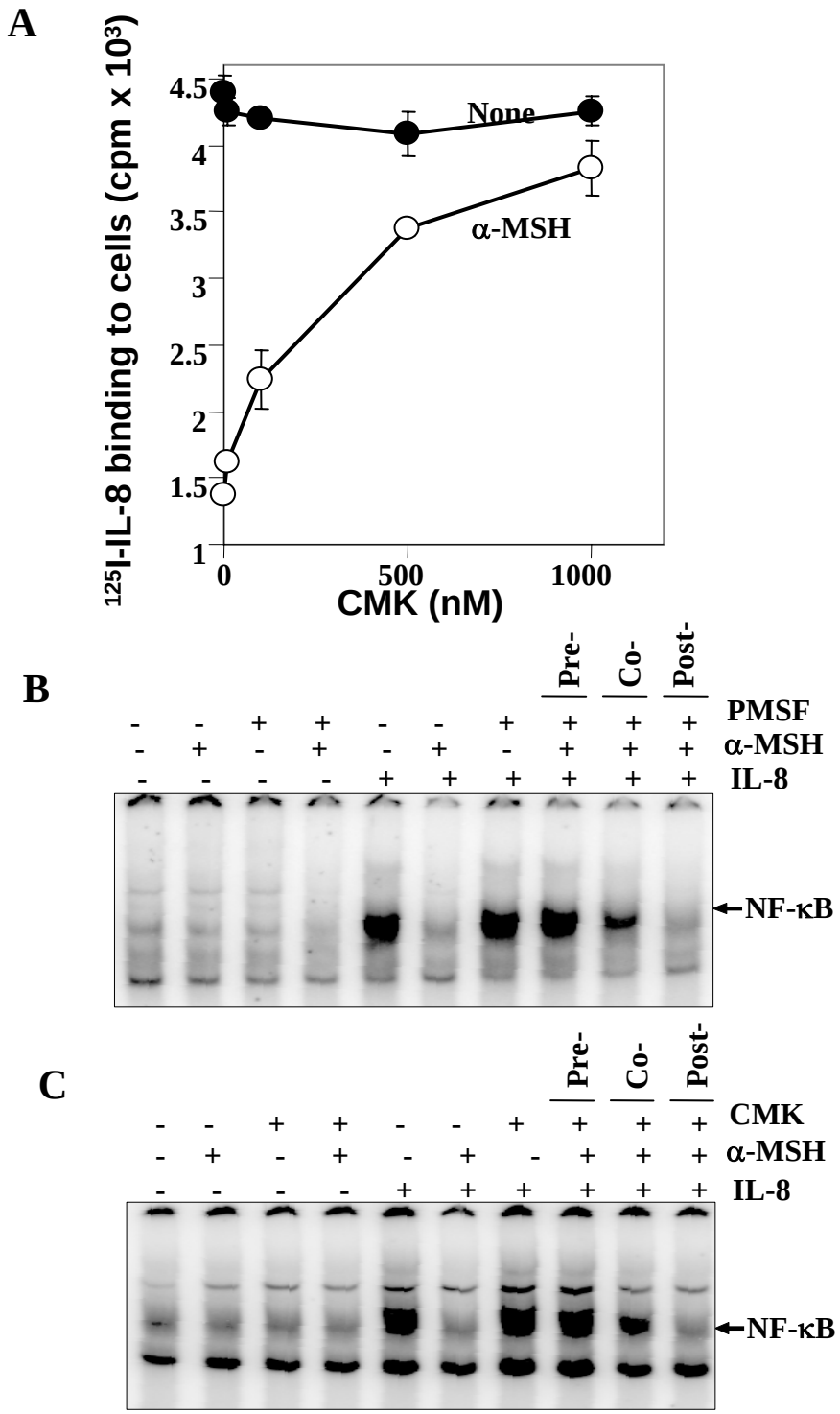


Fig. 5.8.2 A. CMK protects α-MSH-mediated inhibition of IL-8 binding. Neutrophils, pre-incubated with different concentrations of CMK for 2 h were treated with 1000 nM α-MSH for 24 h. ^{125}I-IL-8 binding was carried out at 4^0C as triplicate samples. **B. PMSF, or C. CMK protects α-MSH-mediated inhibition of IL-8-induced NF-kB activation.** Neutrophils were treated with α-MSH for 24 h with pre-, co- or post-treated cells with CMK (1 μM) or PMSF (100 μM). Then cells were stimulated with 100 ng/ml IL-8 for 2 h. Then nuclear extracts were prepared and analyzed for NF-κB by EMSA.

Anti-elastase, but not anti-cathepsin G or anti–proteinase 3 antibodies protects α-MSH-mediated inhibition of IL-8 binding.

To detect the role of different neutrophil proteases induced by α-MSH to downregulate CXCRs, neutrophils, incubated with different antibodies (anti-elastase, -cathepsin G, or -proteinase 3) for 2 h were treated with α-MSH for different times at 37^0C and IL-8 binding was assayed. The Figure 5.9.1A showed that α-MSH decreased IL-8 binding in a dose-dependent manner and anti-elastase, but not by anti-cathepsin G or –proteinase 3 antibody almost completely inhibited α-MSH mediated decrease of IL-8 binding. The result suggests that α-MSH induces elastase in neutrophils, which may downregulate CXCRs.

α-MSH increases neutrophil elastase level and activity.

To detect the role of proteases secreted from α-MSH treated neutrophils, cells were treated with α-MSH (1000 nM) for different times and the culture supernatant was assayed for neutrophil elastase as described in Materials and Methods. The results showed that elastase activity increased with time of α-MSH treatment (Fig.5.9.1.B). Culture supernatant from α-MSH-stimulated cells when incubated with elastase inhibitor, CMK did not show elastase activity indicating its specificity (Scapini *et al.*, 2002). The culture supernatant was concentrated and 100 μg proteins were analyzed for elastase, cathepsin G, and proteinase 3 by Western blot. The level of elastase, but not proteinase 3 or cathepsin G was increased significantly with α-MSH treatment (Fig.5.9.1 C) indicating the possible role of elastase in α-MSH-mediated downregulation of CXCRs.

Neutrophils elastase downregulates IL-8 binding in neutrophils, neutrophils membrane, and affinity purified CXCRs.

To detect the role of elastase on CXCRs, neutrophils, isolated membrane, and affinity purified CXCRs were incubated with 200, 500, and 1000 ng recombinant human neutrophil elastase for 6 h. Then ^{125}I-IL-8 or -TNF binding was assayed from

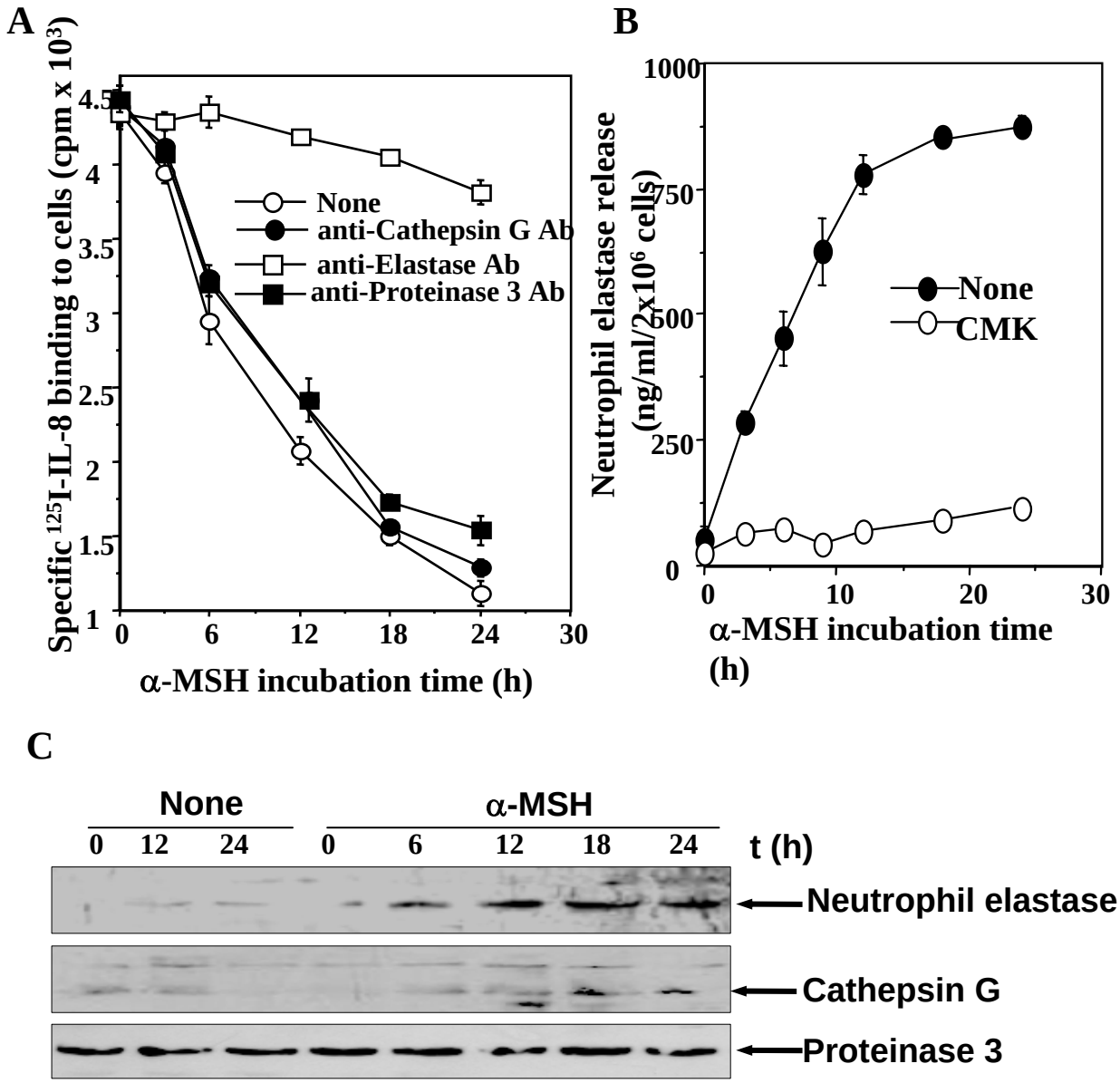


Fig.5.9.1 A. Effect of anti-cathepsin G, -elastase, and –proteinase 3 antibodies on α-MSH mediated downregulation of CXCRs. Neutrophils, pre-incubated with anti-cathepsin G, -elastase, and proteinase 3 (1 μg/μl) for 2 h were treated with α-MSH (1000 nM) for different times and ^{125}I-IL-8 binding was assayed. The result represents one of three independent experiments. **B**. **Effect of α-MSH on elastase release.** Culture supernatants, collected from α-MSH-treated neutrophils were incubated without or with CMK for 2 h and assayed for elastase as described in Materials and Methods. **C**. **Effect of α-MSH on secretion of cathepsin G, elastase, and proteinase 3.** Culture supernatant from α-MSH-treated neutrophils for different times were concentrated (10 times) and 100 μg proteins were used to detect cathepsin G, elastase, and proteinase 3 by Western blot.

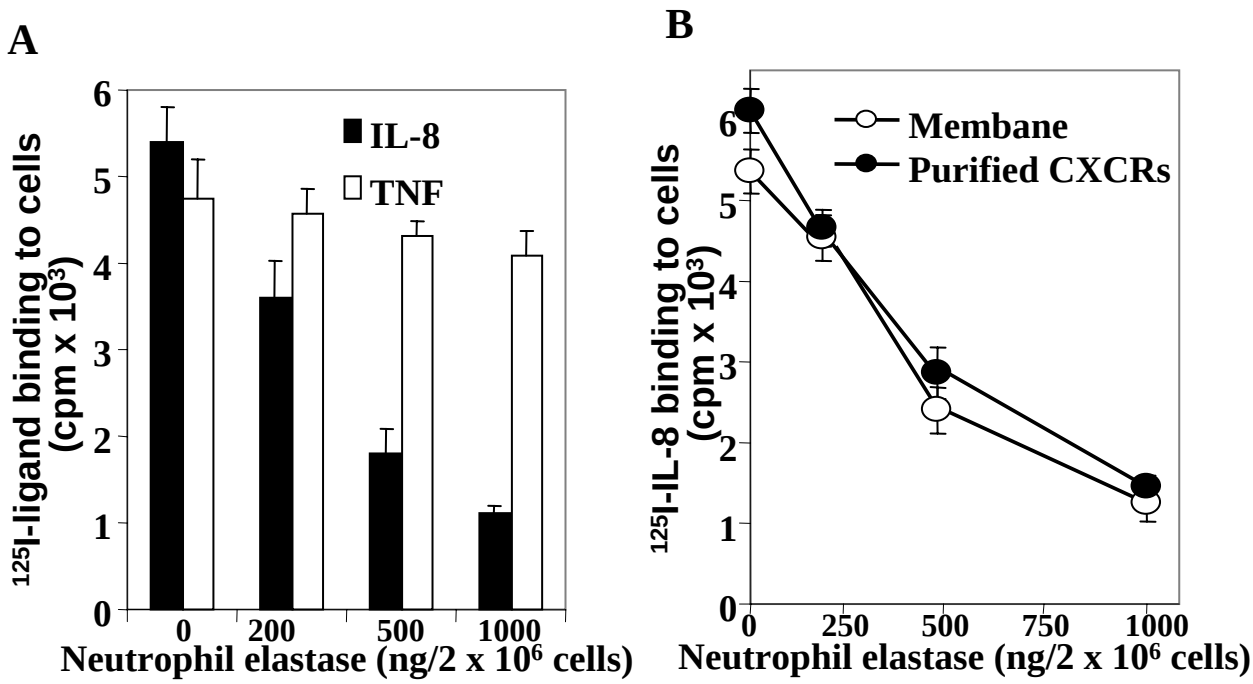


Fig. 5.9.2 Effect of human elastase on IL-8 binding. A. Neutrophils were incubated with 200, 500, and 1000 ng of human neutrophil elastase in triplicate for 6 h. Then ^{125}I-IL-8 or -TNF binding was assayed. **B**. Neutrophil membrane and purified CXCRs were incubated with 200, 500, and 1000 ng of human neutrophil elastase in triplicate for 6 h. Then ^{125}I-IL-8 binding was assayed and mean binding was indicated in figure.

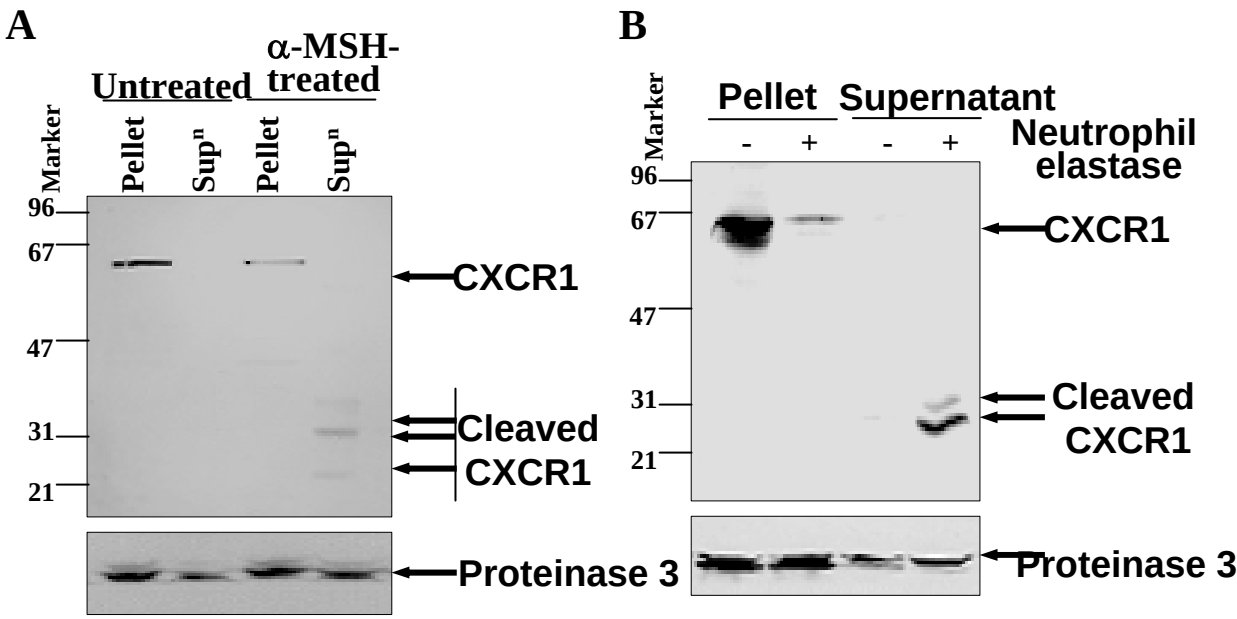


Fig. 5.9.3. A, B. Effect of α-MSH and neutrophil elastase on IL-8 receptor level. Neutrophils were treated with α-MSH (1000 nM) A, for 24 h or neutrophil elastase (500 ng) B, for 6 h. Culture supernatant (100 μg after 10 times concentrated) and pellet extract (200 μg) were assayed for CXCR1 using 15% SDS-PAGE by Western blot. Blots were reprobed with anti-proteinase 3 antibody.

treated neutrophils and IL-8 binding was assayed from membrane, and affinity purified receptors. The IL-8, but not TNF binding was decreased with increasing concentrations of elastase in neutrophils (Fig.5.9.2A). The ^{125}I-IL-8 binding was decreased in purified membrane and CXCRs with increasing concentrations of elastase (Fig.5.9.2 B). The data suggest that elastase downregulates CXCRs.

α-MSH or neutrophil elastase cleaves IL-8 receptor.

To detect the possible cleavage of CXCRs by α-MSH and elastase treatment, neutrophils were treated with 1000 nM α-MSH for 24 h or 500 ng of neutrophil elastase for 6 h. The cell pellet extract (200 μg proteins) and culture supernatant (100 μg) were used to detect CXCR1 by Western blot. The results showed that α-MSH (Fig.5.9.3A) or elastase (Fig.5.9.3B) decreased CXCR1 level in neutrophils and the cleaved bands detected from culture supernatant further suggesting the downregulation of CXCR1s. Both blots were reprobed with anti-proteinase 3 antibody.

α-MSH-mediated downregulation of CXCRs is independent of cAMP.

As α-MSH-mediated cell signaling involves generation of cAMP through activation of adenylate cyclase, neutrophils, pretreated with dideoxyadenosine (ddAdo), an adenylate cyclase inhibitor were treated with α-MSH for 24 h or incubated with dibutyryl cAMP (db cAMP) for 4 h. The level of elastase was detected by Western blot (Fig.5.10A) and colorimetric assay (Fig.5.10B) from culture supernatant. The level of cAMP was detected from cell extract (Fig.5.10 C) and IL-8 binding from neutrophils (Fig.5.10 D). The results showed that α-MSH increased elastase and cAMP levels but decreased IL-8 binding. The ddAdo did not interfere alone but protected α-MSH-mediated downregulation of neutrophil elastase level and IL-8 binding or upregulation of cAMP. Addition of db-cAMP did not increase elastase level or IL-8 binding. These results suggest that α-MSH though increases cAMP level but does not interfere with elastase level or IL-8 binding.

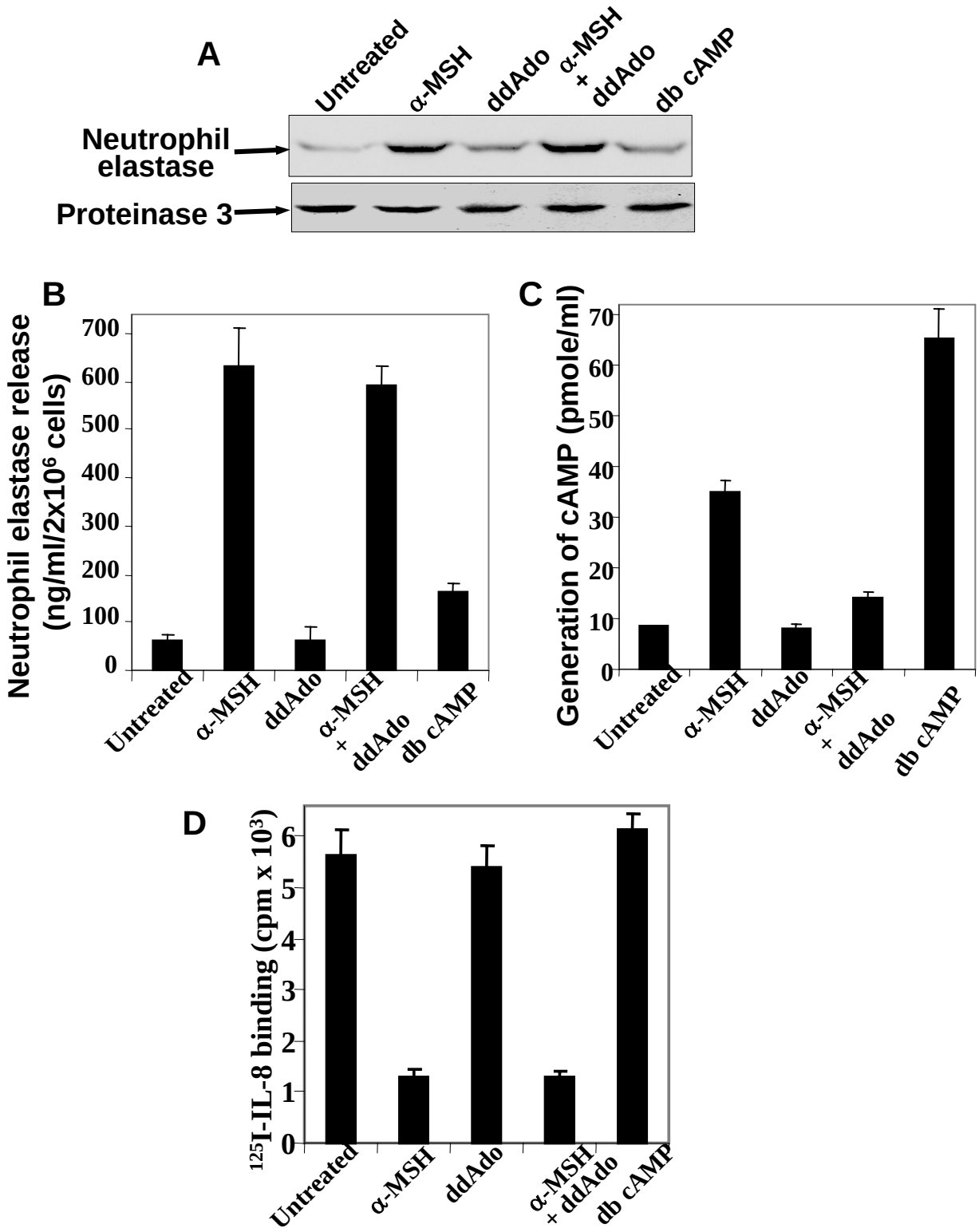


Fig. 5.10. Effect of ddAdo on α-MSH-mediated neutrophil elastase level, cAMP generation, and IL-8 binding. Neutrophils, pretreated with ddAdo (100 mM) for 1 h were treated with α-MSH (1000 nM) for 24 h. In another set, cells were treated with db cAMP (100 mM) for 4 h. From culture supernatant (10 times concentrated) neutrophil elastase level was detected by Western blot (**A**) and activity (**B**) was assayed using substrate. From pellet extracts cAMP was assayed as per manufacturer's protocol as pmole/ml (**C**), and from cells IL-8 binding was assayed (**D**).

α-MSH downregulates IL-8-induced NF-κB activation and proteases release; anti-CXCRs Abs or CMK protects a-MSH-mediated inhibition of IL-8 binding in human blood-derived neutrophils and macrophages.

So far all the experiments described were performed in HL-60-differentiated neutrophils or PMA-differentiated macrophages. Whether α-MSH downregulates CXCRs thereby IL-8-mediated biological responses in neutrophils or macrophages, isolated from fresh human blood was examined. The isolated neutrophils and macrophages showed about 12 and 5.2% cell death respectively at 24 h of culture and cell viability was not altered in presence of CMK. IL-8-induced NF-κB activation was inhibited by α-MSH in a dose-dependent manner in neutrophils (Fig.5.11.1A) and macrophages (Fig.5.11.1B). As shown in Figure 5.11.1C, α-MSH inhibited ^{125}I-IL-8 binding in a dose-dependent manner in both neutrophils and macrophages and this inhibition of IL-8 binding was protected by pre-incubation of anti-CXCRs Abs in neutrophils (Fig.5.11.1D). IL-8-induced myeloperoxidase (Fig.5.11.2A) and β-D-glucuronidase (Fig.5.11.2 B) release in a dose-dependent manner and α-MSH inhibited this release in human neutrophils. CMK protected α-MSH-mediated downregulation of CXCRs in a dose dependent manner in human neutrophils similar to HL-60 differentiated neutrophils as detected by IL-8 binding assay (Fig.5.11.2 C).

5.3 Discussion

Even though several studies indicate that certain neuropeptides, such as α-MSH, have anti-inflammatory effects in different cell types, the mechanism underlying this effect is not understood. As IL-8 is an important inflammatory cytokine and involved in all forms of neutrophil- and macrophage-driven inflammation, it becomes interesting to study the effect of α-MSH on IL-8-mediated neutrophil and macrophage functions.

We have shown that MC-1Rs are predominantly expressed in neutrophils and macrophages. α-MSH inhibited various biological responses generated by IL-8 in

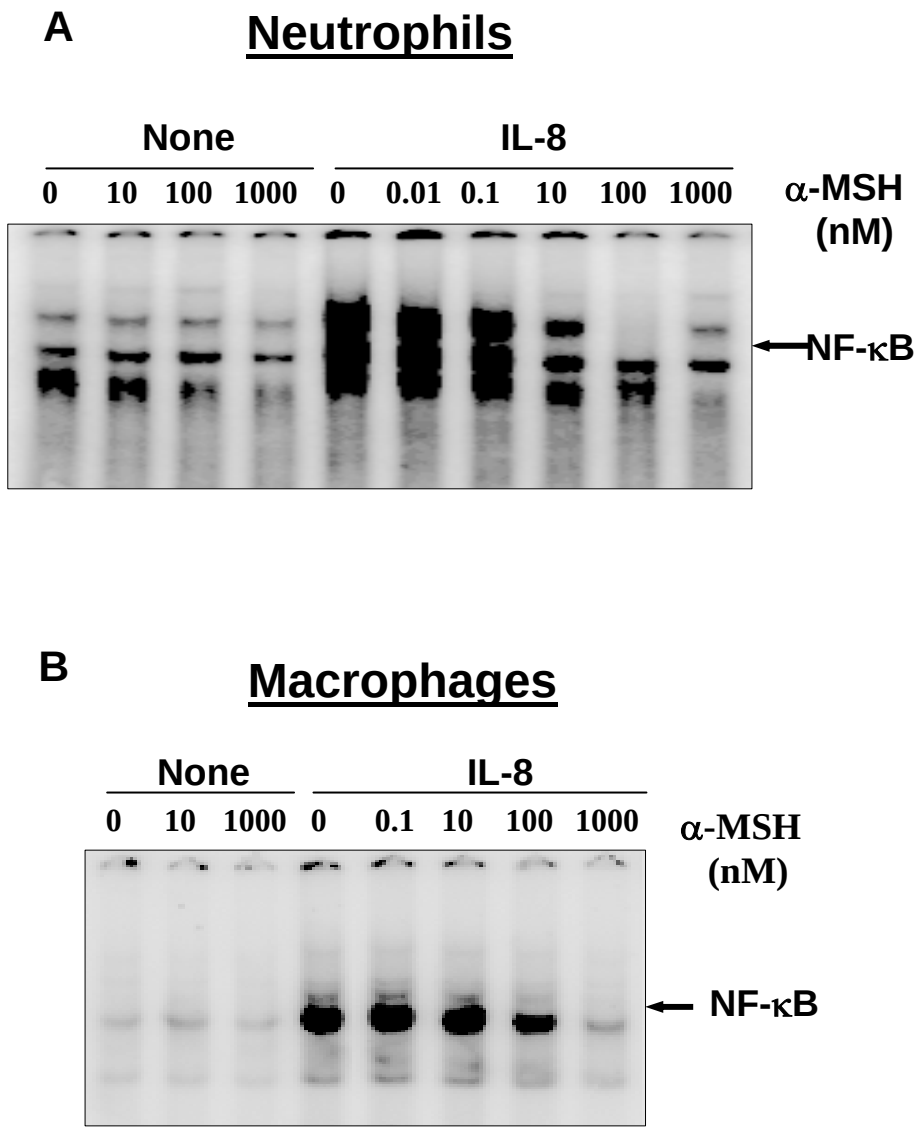


Fig.5.11.1 α-MSH inhibits IL-8-induced NF-κB activation in human neutrophils and macrophages. Neutrophils (**A**) and macrophages (**B**), treated with different concentrations of α-MSH for 24 h were stimulated with IL-8 (100 ng/ml) for 2 h. Nuclear extracts were prepared and NF-κB assayed.

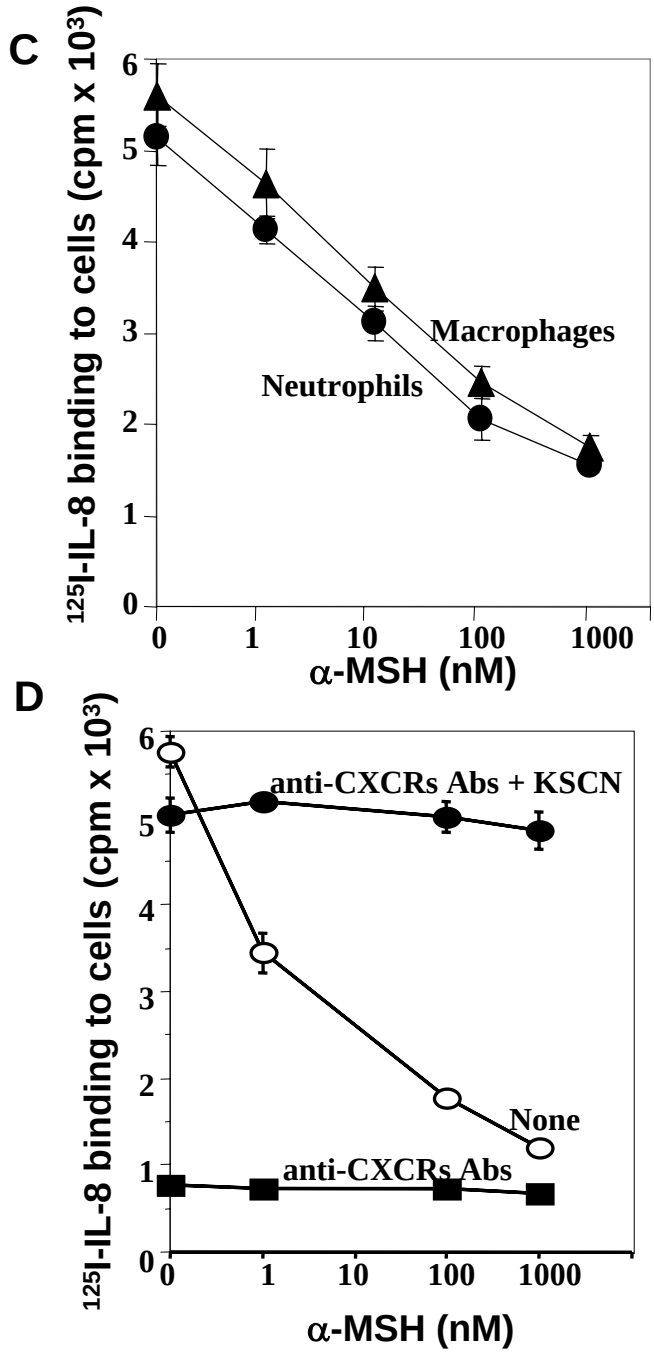


Fig.5.11.1 α-MSH inhibits IL-8 binding in human neutrophils and macrophages. C. Neutrophils and macrophages, isolated from human blood were incubated with different concentrations of α-MSH for 24 h and then IL-8 binding was assayed. **D**. **Anti-CXCRs Abs protect α-MSH-mediated inhibition of IL-8 binding.** Neutrophils, incubated with 1 mg of anti-CXCR1 and R2 Abs for 1 h were treated with different concentrations of α-MSH for 24 h. Cells were washed with KSCN for 10 sec and assayed for 125I-IL-8 binding.

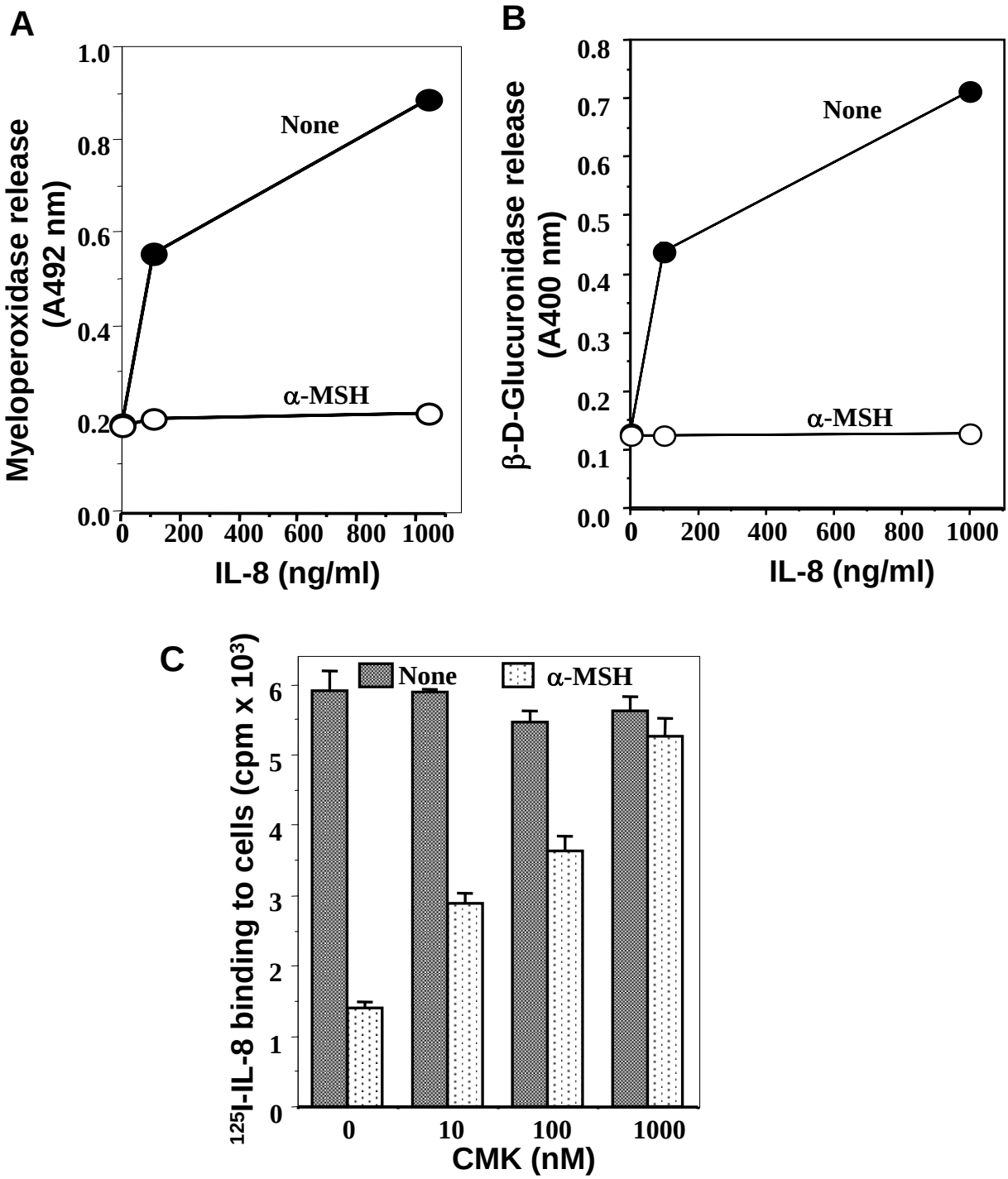


Fig.5.11.2. A, B. α-MSH inhibits IL-8-induced enzymes release. Isolated neutrophils, treated with α-MSH (1000 nM) for 24 h were stimulated with different concentrations of IL-8. Then supernatant was assayed for myeloperoxidase (**A**) and β-D glucoronidase (**B**). **C. CMK protects α-MSH-mediated inhibition of IL-8 binding.** Human neutrophils, pre-treated with different concentrations of CMK for 2 h were treated with α-MSH (1000 nM) for 24 h and then assayed for ^{125}I-IL-8 binding. The result shown is representative of three independent experiments.

neutrophils, such as inhibition of activation of NF-kB (Fig. 5.1), proteolytic enzyme release (Fig.5.2), neutrophil migration (Fig.5.3.1 A) oxidative burst response (Fig. 5.3.2). FMLP is a strong inducer of neutrophil oxidative burst response, but α-MSH did not inhibit FMLP-induced chemotaxis (Fig. 5.3.1A), oxidative burst response (Fig.5.3.2), NF-κB binding, and NF-κB-dependent reporter gene expression indicating its specific function. Downregulated receptors are also detected by chemical cross-linking of labeled ligand-receptor complex (Fig.5.6.2A), immunofluorescent data (Fig. 5.6.3A), and immunoprecipitated CXCRs (Fig.5.6.3B). Downregulation of CXCR1 was also detected from the cleavage fragments from culture supernatant of α-MSH-treated cells. α-MSH downregulated both types of CXCRs as detected by cold competition with MGSA. MGSA binds with CXCR2 with high affinity. Cross-linked CXCRs with ^{125}I-IL-8 showed both bands with equal intensity further prove that the downregulation of both types of CXCRs by α-MSH treatment. The down regulation was at the protein level as there was no change in the mRNA level of CXCRs in presence of α-MSH (Fig. 5.6.4). α-MSH has no effect on CXCRs downregulation in isolated neutrophil membrane or affinity purified CXCRs *in vitro* (Fig.5.7.1A) indicating that α-MSH interacts with viable cells and downregulates CXCRs. The IL-8 or anti-CXCRs Abs protected CXCRs downregulation, suggesting IL-8 or anti-CXCRs Abs may be masking CXCRs and protected IL-8 binding site from α-MSH-mediated modulation. Though MDC protected CXCRs from internalization after binding with IL-8, the unprotected (stored inside the cells in granules as intracellular pool) CXCRs might come out while incubation with α-MSH at 37^0C. As α-MSH downregulated almost 80% CXCRs, these unprotected CXCRs may be not accounted.

PMSF is known to block trypsin- and chymotrypsin-like serine proteases. TPCK and TLCK are chymotrypsin-like and trypsin-like protease inhibitor respectively. PMSF completely protected the α-MSH-mediated CXCRs downregulation indicating the involvement of serine proteases in α-MSH-induced downregulation of CXCRs. CMK, a specific neutrophil elastase inhibitor completely protected CXCRs suggesting involvement of elastase, a serine protease in α-MSH-mediated

downregulation of CXCRs. α-MSH activates cells through interaction of its receptor and signaling to induce or release different proteolytic enzymes from neutrophils as these cells are involved in first line of defense. Though release of neutrophil elastase from neutrophils was increased but the level of myeloperoxidase was not increased significantly by α-MSH treatment. Elastase and myeloperoxidase retain in the primary granules (Kato *et al.,* 2004**)**, but how α-MSH specifically induces neutrophils to release elastase needs to be further studied. The neutrophil elastase is reported to shed TNFRs from cell surface and about 6 μg/ml neutrophil elastase decreases 50% TNF binding in neutrophils (Porteu *et al.,* 1991**).** We did not detect significant downregulation of TNFRs. The level of neutrophils elastase released due to 1000 nM α-MSH treatment was probably to less to decrease the TNFRs. Neutrophil elastase downregulated CXCRs in neutrophils (which cleaves CXCR1 in to smaller fragments), isolated membrane, and affinity purified CXCRs *in vitro*. α-MSH mediated downregulation of CXCRs followed by IL-8-induced biological responses as shown in isolated human neutrophils and macrophages (Fig.5.11.1).

Migrated neutrophils and residential macrophages release proteases not only to clear the microbes in case of infection but also to destroy the surrounding tissues during inflammation. α-MSH releases specific protease from neutrophils, which specifically downregulates CXCRs thus regulating excessive migration of neutrophils towards the inflamed tissues in case of neutrophil-driven inflammatory diseases and hence may help to ameliorate the suffering of such patients.

CHAPTER SIX

MANGIFERIN, A BETA-D-GLUCOSIDE SUPPRESSES TNF-INDUCED CELL SIGNALING

6.1 Introduction

Phenolic antioxidants are well known to exhibit anti-inflammatory, anti-carcinogenic and anti-diabetic activities in animals (Futakuchi *et al.,* 1998; Talalay *et al.,* 1998; Wattenberg *et al.,* 1980). Human beings consume antioxidants from dietary sources, either as natural components or as synthetic food additives (Yang *et al.,* 2001). The broad spectrum of the function of phenolic antioxidants suggests their multiple targets through which they interfere with various cellular functions and protect against pathological lesions such as cancer and inflammatory diseases. Currently there is an increasing interest in therapeutic use of antioxidants to prevent tissue damage induced by overproduction of ROI, by reducing free radical formation or by scavenging or promoting the breakdown of these species (Young and Woodside, 2001, Coliins AR, 1999; Cuzzocrea *et al.,* 2001). Experiments in different *in vitro* and *in vivo* systems have demonstrated the potent anti-oxidant action of plant polyphenol (Damianaki *et al.,* 2000), and it has been suggested that they can prevent oxidative-stress related diseases (Aucamp *et al.,* 1997).

Recently, the polyphenol mangiferin, a C-glucosylxanthone, specifically 1,3,6,7-hydroxyxanthone-C2-β-D-glucoside, has attracted considerable interest in view of its numerous pharmacological activities, including antitumor, antiviral (Guha *et al.,* 1996; Yoshimi *et al.,* 2001), antidiabetic (Miura *et al.,* 2001), anti-bone resorption (Li *et al.,* 1998), and anti-oxidant activity (Moreira *et al.,* 2001). The anti- inflammatory property is reported to be the consequence of inhibition of inflammatory regulators like COX-2, PGE2, NF-κB, TNF-α. (Leiro *et al.,* 2004 A; Leiro *et al.,* 2004 B). Mangiferin is shown to form complex with Iron thereby modulating the mitochondrial lipid peroxidation (Andreu *et al.,* 2005). Mangiferin is also shown to cause apoptosis of leukemic cell line K562 by repression of bcr/abl gene expression. Mangiferin is already being produced on an industrial scale in Cuba to be used as a nutritional supplement (Nunez Selles *et al.,* 2002). However, the mechanisms involved in mangiferin-mediated actions are not clearly understood.

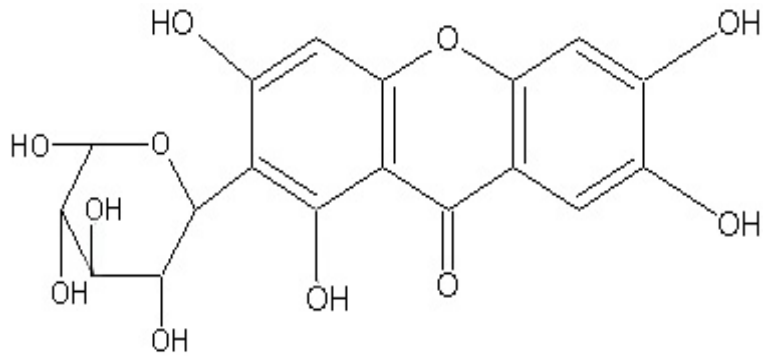
Keeping in mind the beneficial effects exhibited by mangiferin and with the increasing demand in the market for drugs with high therapeutic efficacy but least side effects along with knowledge of its mechanism of action, we chose to study the signaling pathway of mangiferin.

6.2 Results

We examined the effect of mangiferin on TNF-induced signal transduction. The chemical structure of mangiferin is shown in Fig.6.1. As U-937 cells express both types of TNF receptor, and as TNF-induced responses in this cell type are well characterized in our laboratory, these cells were used for our studies. Mangiferin was dissolved in DMSO (10 mg/ml) and further dilutions were made in complete medium. At the concentration of mangiferin and duration of exposure employed in these studies, there was no effect on cell viability as detected by Trypan blue dye exclusion method (98.52 ± 2.48, 96.62 ± 4.82, and 96.24 ± 3.76 percentage of cell viability was observed at 2, 5, and 10 μg/ml mangiferin respectively for 3 h of incubation).

Inhibition of TNF induced NF-κB activation by mangiferin.

U-937 cells were pretreated for 3 h with different concentrations (0-10 μg/ml) of mangiferin and then stimulated with and without 0.1nM TNF for 30 min. Nuclear extracts were prepared and assayed for NF-κB by EMSA. As shown in Fig. 6.2.1, Mangiferin inhibited TNF induced activation of NF-κB, in a dose-dependent manner; with a complete inhibition at 10 μg/ml. To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated the nuclear extracts from TNF-activated cells with antibody to either p50 (NF-κBI) or p65 (Rel A) subunits and then performed EMSA. Antibodies to either subunit of NF-κB shifted the band to a higher mol.wt. (Fig. 6.2.2), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant antibodies such as anti-c-Rel or anti-cyclinD1 had any effect on the mobility of NF-κB. Excess cold NF-κB (100-fold) almost completely eliminated the band, indicating the specificity of NF-κB.



Mangiferin
(1,3,6,7 tetrahydroxy C2 beta-D-glucoside)

Fig.6.1. Structure of mangiferin

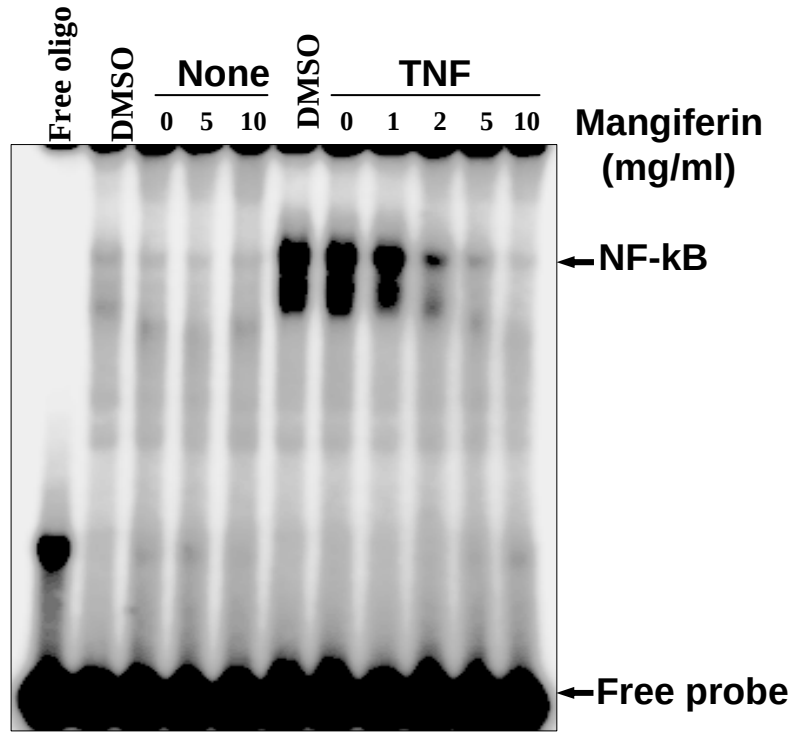


Fig. 6.2.1. Effect of mangiferin on of TNF-dependent NF-κB activation. U-937 cells (2×10^6/ml) were preincubated at 37°C for 3 h with different concentrations of mangiferin, followed by 30-min incubation with 0.1 nM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB, as described in materials and methods.

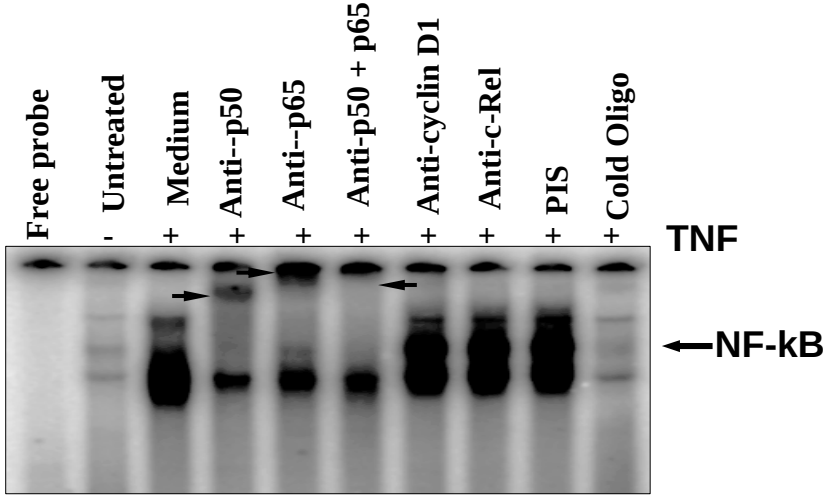


Fig.6.2.2. Supershift and specificity of NF-κB activation. Nuclear extracts, prepared from untreated or TNF-treated U-937 cells were incubated for 15 min with different Abs and cold NF-κB oligonucleotide, and then assayed for NF-κB, as described in materials and methods.

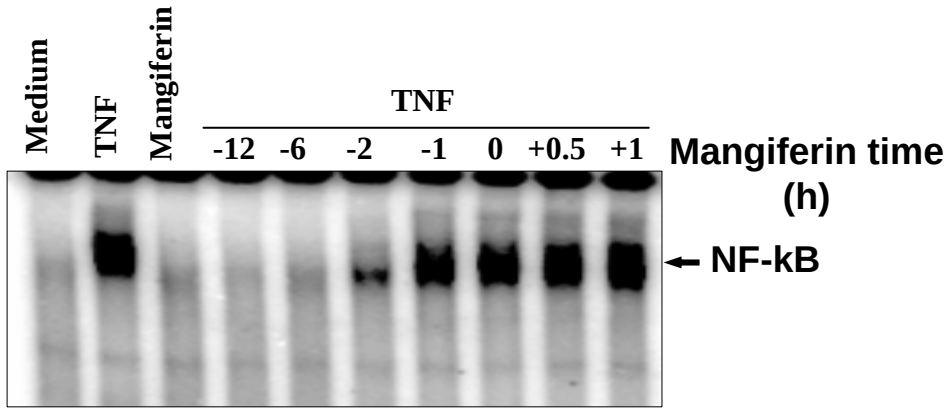


Fig.6.2.3. Effect of Pre-Co and Post treatment of mangiferin. Cells, either pre-, co-, or post-incubated with 10 µg/ml mangiferin at 37°C for the indicated times were stimulated with 0.1 nM TNF at 37°C for 30 min. After these treatments nuclear extracts were prepared and then assayed for NF-κB.

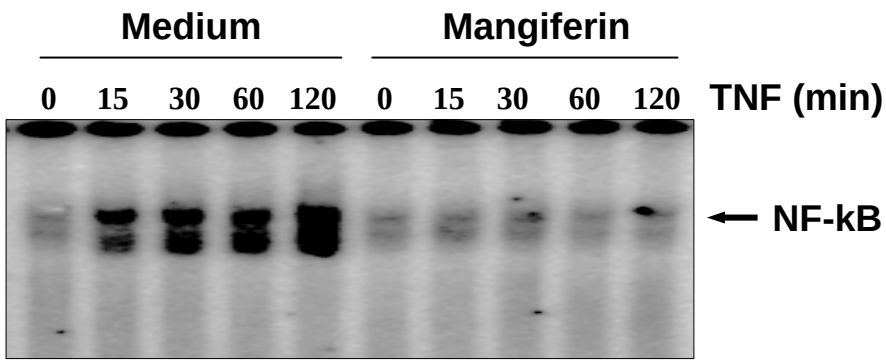


Fig.6.2.4.Time course of inhibition of TNF-dependent NF-κB activation by mangiferin. U-937 cells (2×10^6/ml), were incubated at 37^0C either with or without 10 µg/ml of mangiferin for 3 h and then stimulated with 0.1 nM TNF for different times. After these treatments nuclear extracts were prepared and then assayed for NF-κB.

Mangiferin by itself did not activate NF-κB. However, it was unable to protect the activation of NF-κB when given as post treatment of TNF (Fig 6.2.3). We also examined the effect of mangiferin on the kinetics of TNF-induced NF-κB activation. Both untreated and mangiferin-pretreated cells were incubated with TNF (0.1nM) for different times and then assayed for NF-κB. In untreated cells, TNF activated NF-κB in a time-dependent manner with almost maximum activation at 15 min. In mangiferin-pretreated cells, activation of NF-κB was inhibited at all time points of TNF stimulation (Fig. 6.2.4).

Mangiferin represses expression of TNF-induced NF-κB-dependent reporter gene.

Although we have shown by EMSA that mangiferin blocks the NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting the role of additional regulatory mechanisms (Nasuhara *et al.,* 1999). To determine the effect of mangiferin on expression of TNF-induced NF-κB-dependent reporter gene, we transiently co-transfected U-937 cells with the *NF-κB SEAP* reporter construct and *β-galactosidase,* then treated with mangiferin (10 μg/ml) for 3 h. Cells were then stimulated with different concentrations of TNF for 12 h. TNF-induced SEAP activity was almost completely abolished when the cells were pretreated with mangiferin (Fig.6.3.1). These results demonstrate that mangiferin also represses NF-κB-dependent reporter gene expression induced by TNF.

Mangiferin inhibits expression of TNF-induced ICAM1, COX2 and COX2 dependent reporter gene.

As mangiferin inhibited TNF-induced different biological responses, we examined the expression of NF-κB regulated genes, adhesion molecule ICAM1 and COX2. U-937 cells were treated with different concentrations of mangiferin for 3 h and then stimulated with 0.1 nM TNF for 12 h. We analyzed ICAM1 and COX2 in the cell extract proteins by Western blot. Expression of TNF induced ICAM1 (Fig.6.3.2 A) and COX-2 (Fig.6.3.2 B) was decreased with increasing concentrations of mangiferin

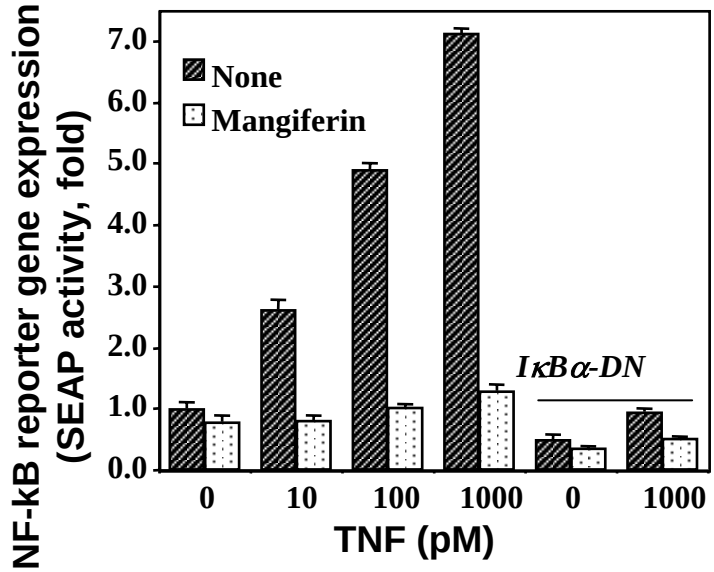


Fig.6.3.1. Mangiferin inhibits the NF-κB-dependent reporter gene expression induced by TNF. U-937 cells were transiently co-transfected with NF-κB responsive promoter linked to SEAP, IκBα-DN, and β-galactosidase genes. After 12 h of transfection, cells were treated with 10 μg/ml mangiferin for 3 h and then stimulated with different concentrations of TNF for additional 12 h. Culture supernatant was collected and assayed for SEAP and pellet was extracted and assayed for β-galactosidase.

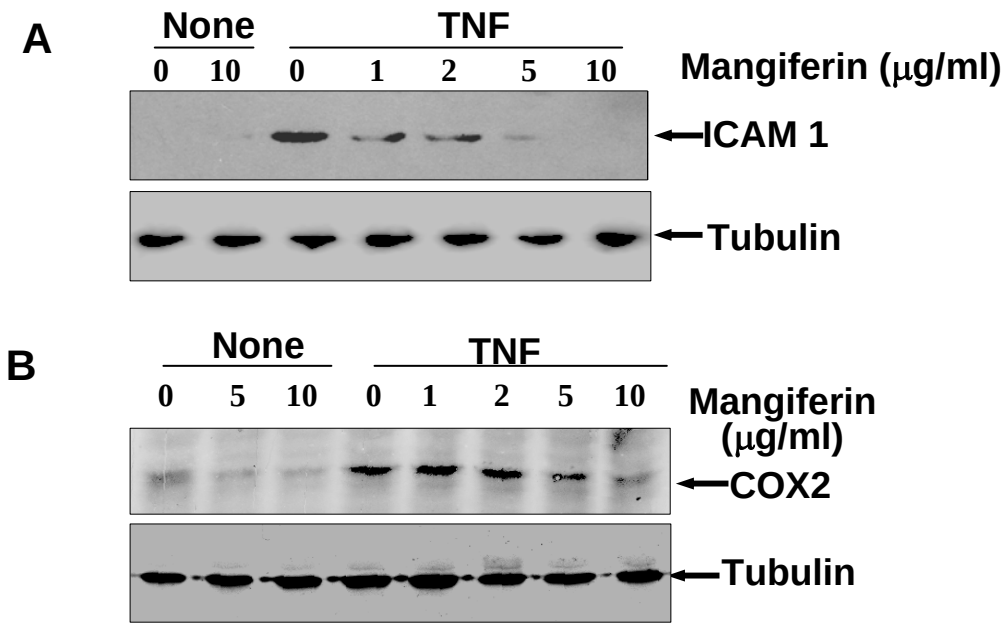


Fig.6.3.2. Effect of mangiferin on TNF induced expression of ICAM1 and COX2. Cells were stimulated with different concentrations of mangiferin for 3 h and then treated with 100 pM of TNF for 12 h at 37^0C in a CO_2 incubator. ICAM1 (**A**) and COX2 (**B**) were analyzed from cell extract (100 μg) by Western blot technique. As loading control, Tubulin was detected by re-probing the same blot.

treatment. Mangiferin alone did not induce expression of ICAM1 or COX2 at 10 μg/ml concentration. Upon re-probing the gels with anti-tubulin antibody, we found that the band intensities in all lanes were uniform indicating equal loading of extracted protein in the lanes.

Although we have shown that mangiferin blocks the expression of COX2, the dependent gene transcription was also assayed. To determine the effect of mangiferin on TNF-induced COX2 dependent reporter gene expression, we transiently transfected U-937 cells with the *COX2-Luciferase* reporter construct and then treated with different concentrations of mangiferin for 3 h. Cells were then stimulated with TNF-α (0.1 nM) for 12 h. TNF-induced activity of luciferase was almost completely abolished when the cells were pretreated with 10 μg/ml mangiferin (Fig.6.3.3). These results demonstrate that mangiferin also represses COX2-dependent reporter gene expression induced by TNF. To further prove that COX2 is indeed NF-κB-dependent gene product, which is inhibited by mangiferin, we transiently transfected U-937 cells with *COX2-Luciferase*, *p65*, and *NF-κB-SEAP* constructs. As mentioned earlier, TNF induced NF-κB and SEAP activity in vector and *COX2-Luciferase* transfected cells, which was inhibited by mangiferin pretreatment. However, mangiferin was unable to inhibit the NF-κB and thereby the SEAP activity in p65-overexpressed cells (Fig.6.3.4 A & 6.3.4 B). To show the link between COX2 and NF-κB, the *COX2-Luciferase* was also assayed in the same set of experiments. The data proved that TNF-induced NF-κB activates COX2, which then drives the expression of its reporter luciferase activity and this was inhibited by mangiferin pretreatment. In p65-overexpressed cells, COX2 protein level increased compared to vector transfected cells, which correlated with the expression of luciferase and its activity (Fig.6.3.4 C & 6.3.4 D). However mangiferin did not inhibit luciferase activity in p65 overexpressed cells as shown in SEAP expression. Since the expression of p65 has led to the expression of COX2, it is clear that COX2 is a NF-κB-dependent gene product.

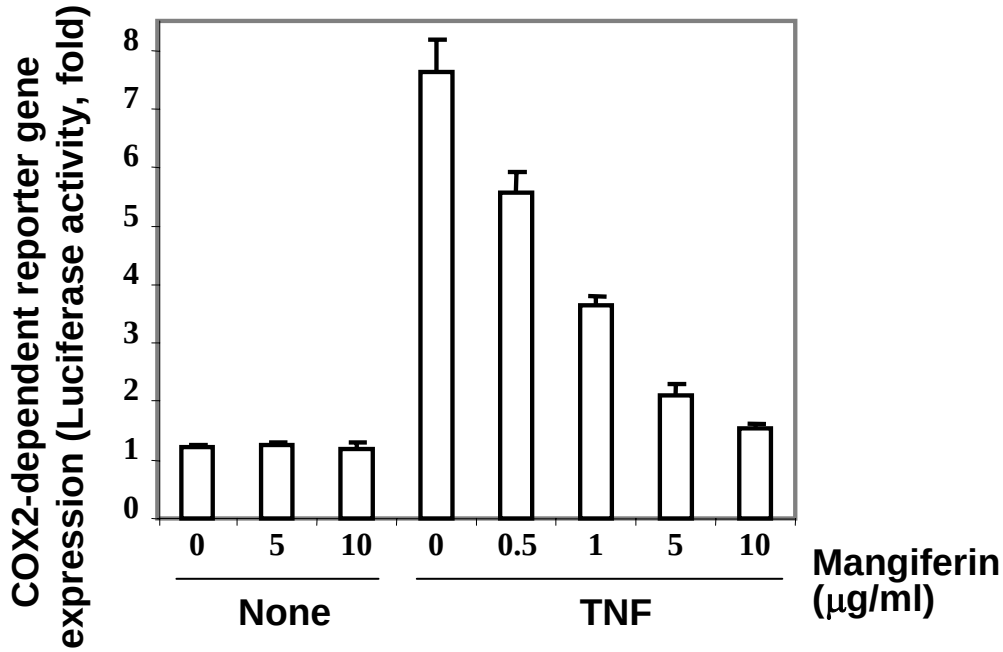


Fig.6.3.3. Mangiferin inhibits COX2 induced gene expression. U-937 cells were transfected with the *COX2-Luciferase* expression vector. After 12 h of transfection, cells were treated with different concentrations of mangiferin for 3 h and then stimulated with 0.1 nM TNF for 12 h. The luciferase activity was measured as described in materials and methods.

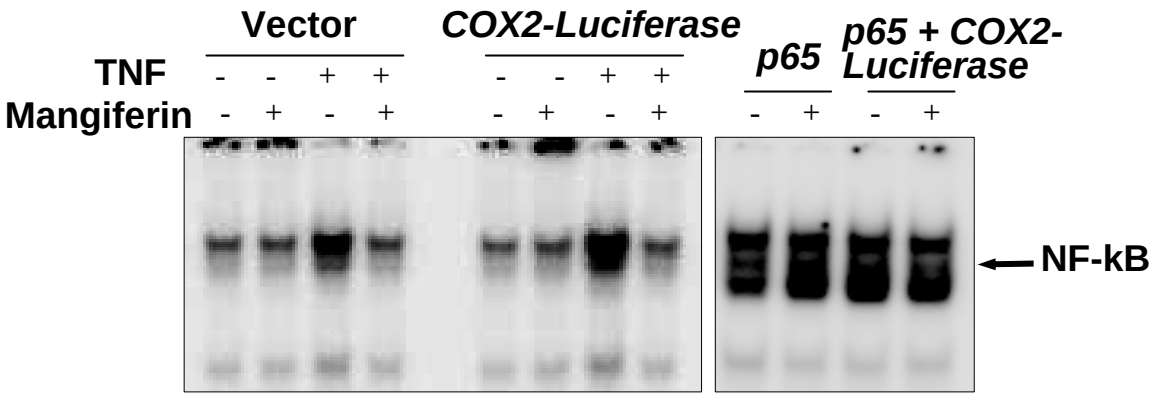


Fig.6.3.4. Involvement of p65 on COX2 gene expression. U-937 cells were co-transfected with the *COX2-Luciferase, p65,* or in combination with *NF-kB-SEAP* reporter construct. After 12 h of transfection, cells were treated with 10 μg/ml mangiferin for 3 h and then stimulated with 0.1 nM TNF for 12 h. Nuclear extracts were analyzed for NF-kB by EMSA.

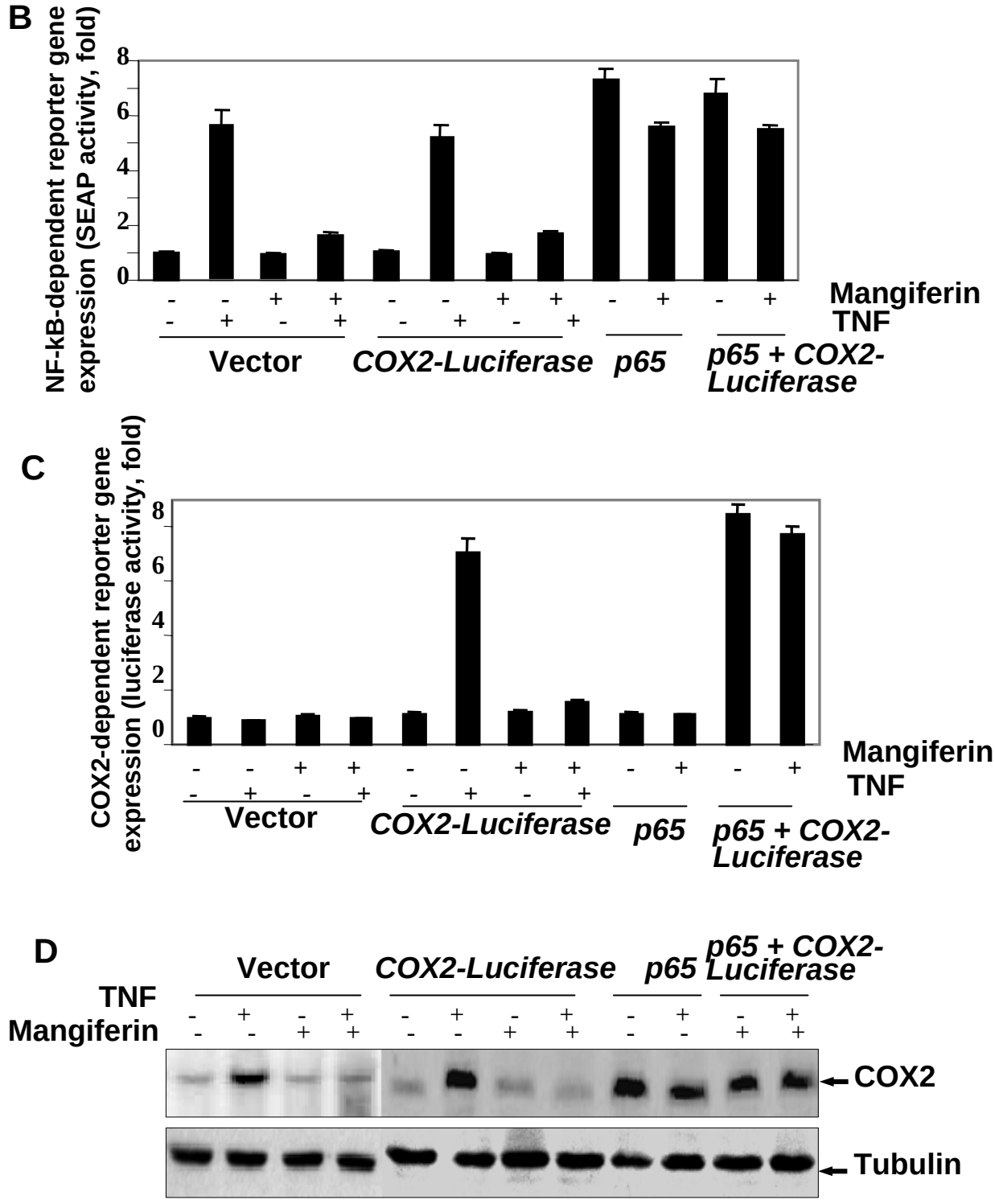


Fig.6.3.4. Involvement of p65 on COX2 gene expression. U-937 cells were co-transfected with the *COX2-Luciferase*, *p65*, or in combination with *NF-κB-SEAP* reporter construct. After 12 h of transfection, cells were treated with 10 μg/ml mangiferin for 3 h and then stimulated with 0.1 nM TNF for 12 h. Culture supernatant was assayed for SEAP activity (**B**), whole cell extracts were assayed for luciferase activity (**C**) and the nuclear extracts were assayed for DNA binding by NF-κB (**D**)

Mangiferin inhibits TNF-dependent phosphorylation and degradation of IκB-α.

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα (Coliins AR, 1999). To determine whether the inhibitory action of mangiferin was due to an effect on IκBα degradation, the cytoplasmic level of IκBα proteins was examined by Western blot analysis. IκBα degradation started at 15 min of TNF (0.1 nM) treatment in U-937 cells and was complete within 30 min. The band reappeared by 120 min owing to NF-κB-dependent IκBα resynthesis. The presence of mangiferin inhibited the TNF-induced IκBα degradation (Fig.6.4.1). To determine whether mangiferin modulates TNF-induced IκBα phosphorylation, Western blot with antibodies against the phosphorylated form of IκBα was performed. Mangiferin was found to inhibit TNF-induced phosphorylation of IκBα (Fig 6.4.1).

As IκBα phosphorylation is required for IKK activation, the effect of mangiferin on IKK activation was examined. U-937 cells were pre-treated with mangiferin (10μg/ml) for 3 h and then stimulated with 0.1 nM TNF for different times. The cell extracts were prepared and IKK activation was detected *in vitro* using GST-IκBα as substrate protein as described in materials and methods. The GST-IκBα specific signal was increased with time of TNF incubation whereas mangiferin pre-treatment completely suppressed the bands at all time points (Fig.6.4.2) indicating mangiferin's role on suppression of TNF-induced IKK activation. 50 μg of extract proteins were analyzed in SDS-PAGE (10%) and probed with anti-IKKα antibody. The intensity of bands was equal in all lanes suggesting equal expression of IKKα. To study whether mangiferin has direct interaction with IKK, cells extracts from TNF-induced cells were incubated with different concentrations of mangiferin in cell-free system and then IKKα was assayed using those extracts. The intensity of bands was equal in all the lanes treated with different concentrations of mangiferin (Fig.6.4.2) suggesting that in cell-free system mangiferin has no role to inhibit IKK activity.

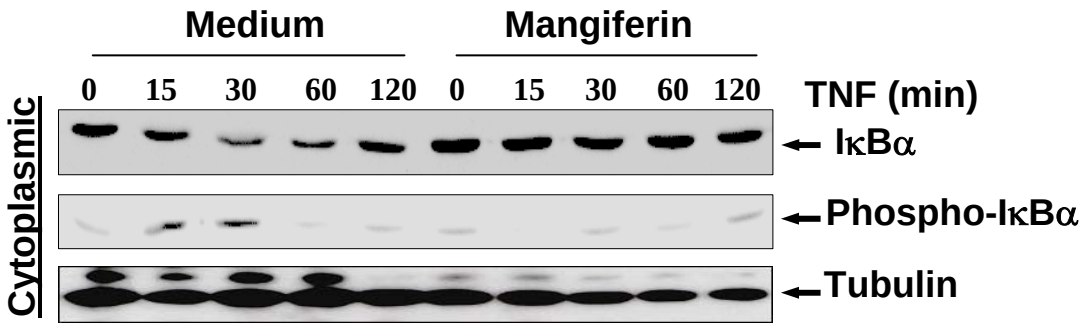


Fig.6.4.1. Effect of mangiferin on TNF-induced degradation of IκBα and on levels of phospho IκB, p65 and phospho p65. U937 cells (2×10^6/ml), either untreated or pretreated with 10 μg/ml mangiferin for 3 h were stimulated with TNF (0.1 nM) for different times. Cytoplasmic extracts were prepared and assayed for IκBα and phospho IκBα in cytosolic fractions by Western blot analysis.

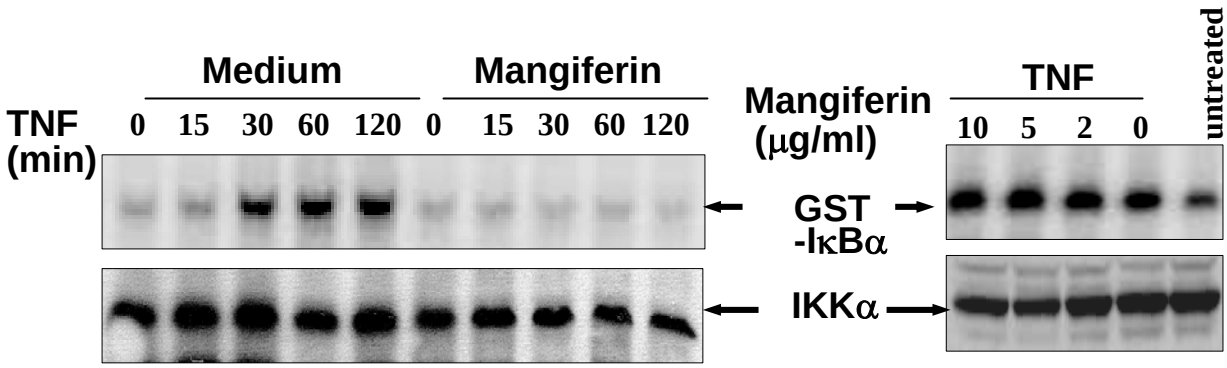


Fig.6.4.2. Effect of mangiferin on the TNF-induced activation of IKK-α. Cells were stimulated with different concentrations of mangiferin followed by 0.1 nM TNF for 1 h and 250 μg whole cell extract proteins were immunoprecipitated with anti-IKK-α antibody. Thereafter immunocomplex kinase assay was performed using GST-IκB-α as a substrate as described in materials and methods. To examine the effect of mangiferin on the level of expression of IKK-α, 50 μg cells extract proteins were analyzed in 10% SDS-PAGE and assayed for IKK-α by Western blot.

Mangiferin inhibits TNF-induced phosphorylation and translocation of p65 subunit of NF-κB.

We wanted to study if mangiferin pretreatment affected the p65 phosphorylation in TNF induced cells. Upon TNF treatment, p65 level was increased in the nucleus with time whereas in mangiferin-pretreated cells, TNF was unable to increase the p65 level with time (Fig.6.4.3 A). The level of phospho-p65 was increased with incubation time of TNF in the nucleus, whereas mangiferin completely inhibited TNF-induced p65 phosphorylation at all time points (Fig. 6.4.3 B). Mangiferin alone had no effect in these experiments. These results indicate that mangiferin blocks the nuclear translocation of p65. Inhibition of p65 translocation by mangiferin was also proved by immunofluorescence wherein mangiferin pretreated cells did not show p65 signal, otherwise shown by TNF alone treated cells, in the nucleus (Fig 6.4.4).

Mangiferin blocks PMA-, LPS-, ceramide induced activation of NF-κB.

Besides TNF, NF-κB is also activated by various other tumor promoters and inflammatory agents, including phorbol myristate acetate (PMA), serum activated LPS (SA-LPS) and ceramide (Baeuerle and Baichwal, 1997) by different signal transduction pathways (Bonizzi *et al,* 1997; Li and Karin, 1998; Imbert *et al.,* 1996). We found that these agents activated NF-κB and that mangiferin completely blocked the activation of NF-κB induced by PMA, SA-LPS, ceramide (Fig.6.5). These results suggest that mangiferin may act at a step in which all these agents converge in the signal transduction pathway leading to NF-κB activation.

Inhibition of NF-κB activation by mangiferin is not cell type specific.

As NF-κB activation pathways differ in different cell types, we therefore studied whether mangiferin affects other cell types as well. It has been demonstrated that distinct signal transduction pathways could mediate NF-κB induction in epithelial and lymphoid cells (Bonizzi *et al.,* 1997). All the effects of mangiferin described above were conducted with U-937, a human histiocytic lymphoma. In another set of experiments, we found that mangiferin blocks TNF-induced NF-κB activation in

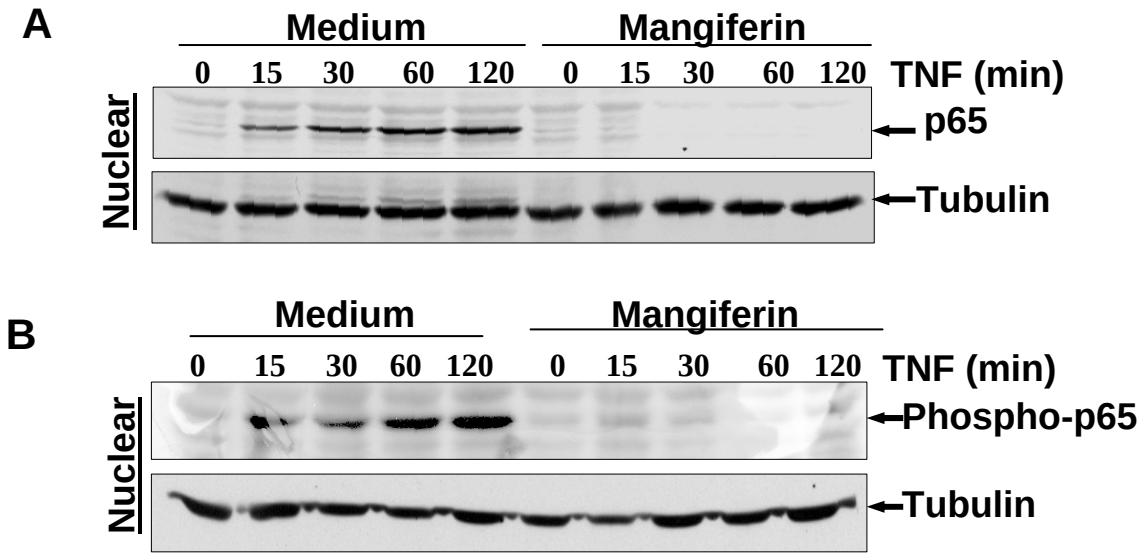


Fig.6.4.3. Effect of mangiferin on TNF-induced p65 and p65 phosphorylation. U937 cells (2×10^6/ml), either untreated or pretreated with 10 μg/ml mangiferin for 3 h were stimulated with TNF (0.1 nM) for different times. Nuclear extracts were prepared and assayed for p65 (**A**) and phospho p65 (**B**) by Western blot analysis. The blots were reprobed with tubulin to show equal loading.

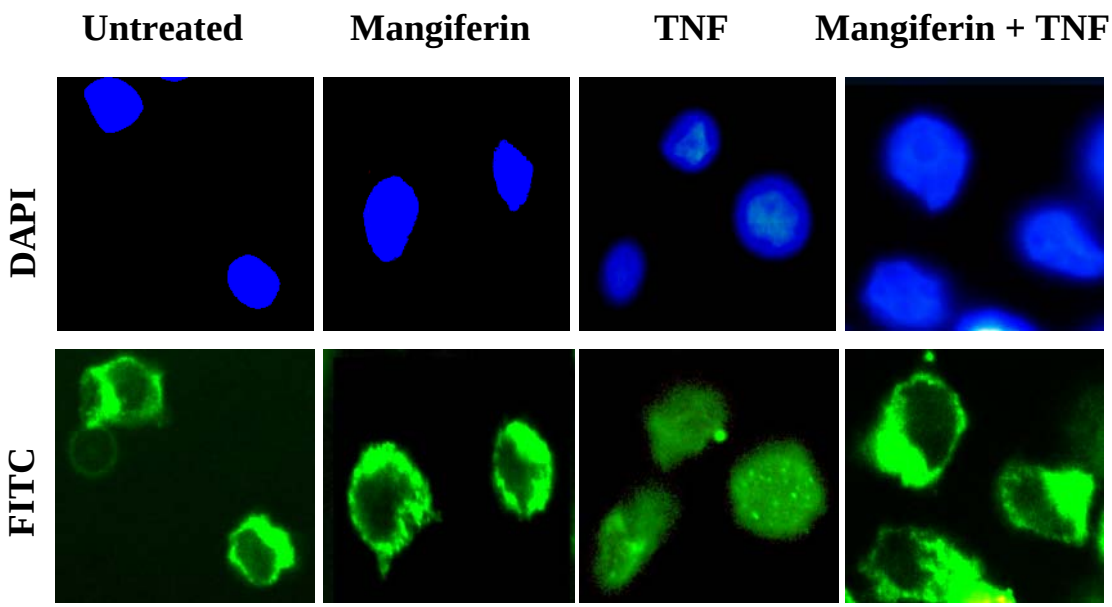


Fig.6.4.4. Mangiferin inhibits TNF-induced nuclear translocation of p65. U-937 cells were treated with or without mangiferin (10 μg/ml) and then stimulated with TNF (0.1 nM) for 30 min. Cells were subjected to immunocytochemistry as described under materials and methods.

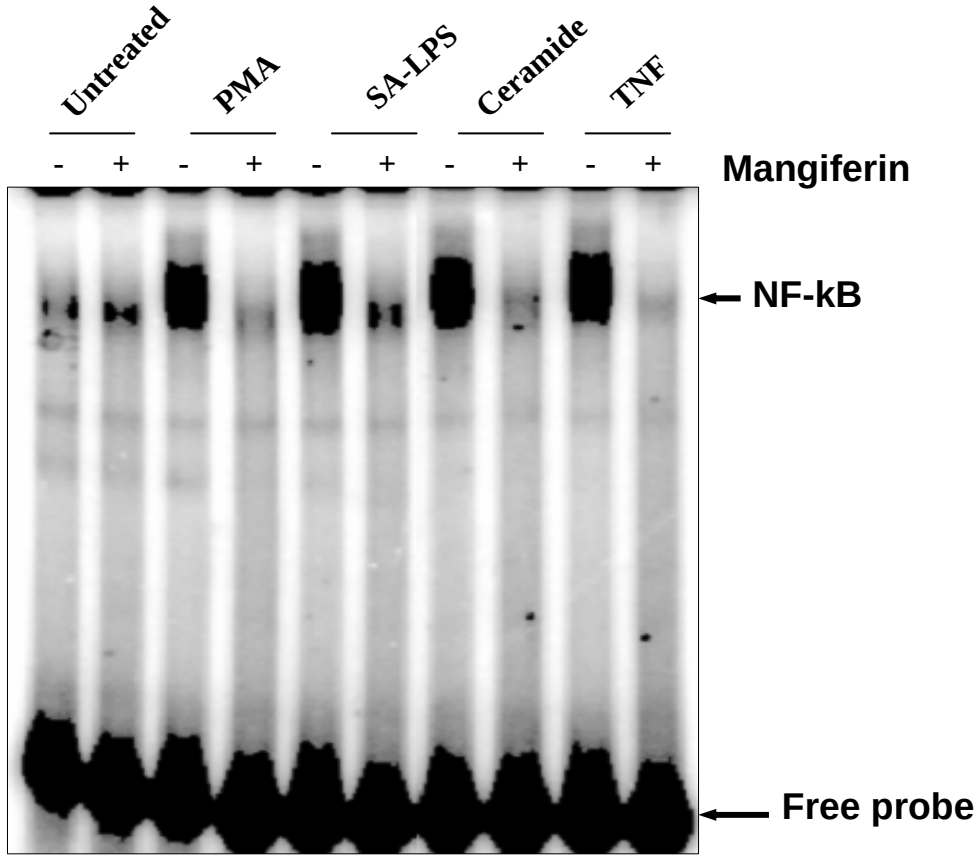


Fig.6.5. Effect of mangiferin on PMA-, serum-activated LPS-, ceramide-, and TNF-induced NF-κB activation. U-937 cells (2×10^6/ml) were treated with mangiferin (10 µg/ml) for 3 h at 37°C followed by stimulation with PMA (25 ng/ml), serum-activated LPS (100 ng/ml), ceramide (10 µM), and TNF (0.1 nM) for 30 min. NF-κB activation was assayed by EMSA from nuclear extract.

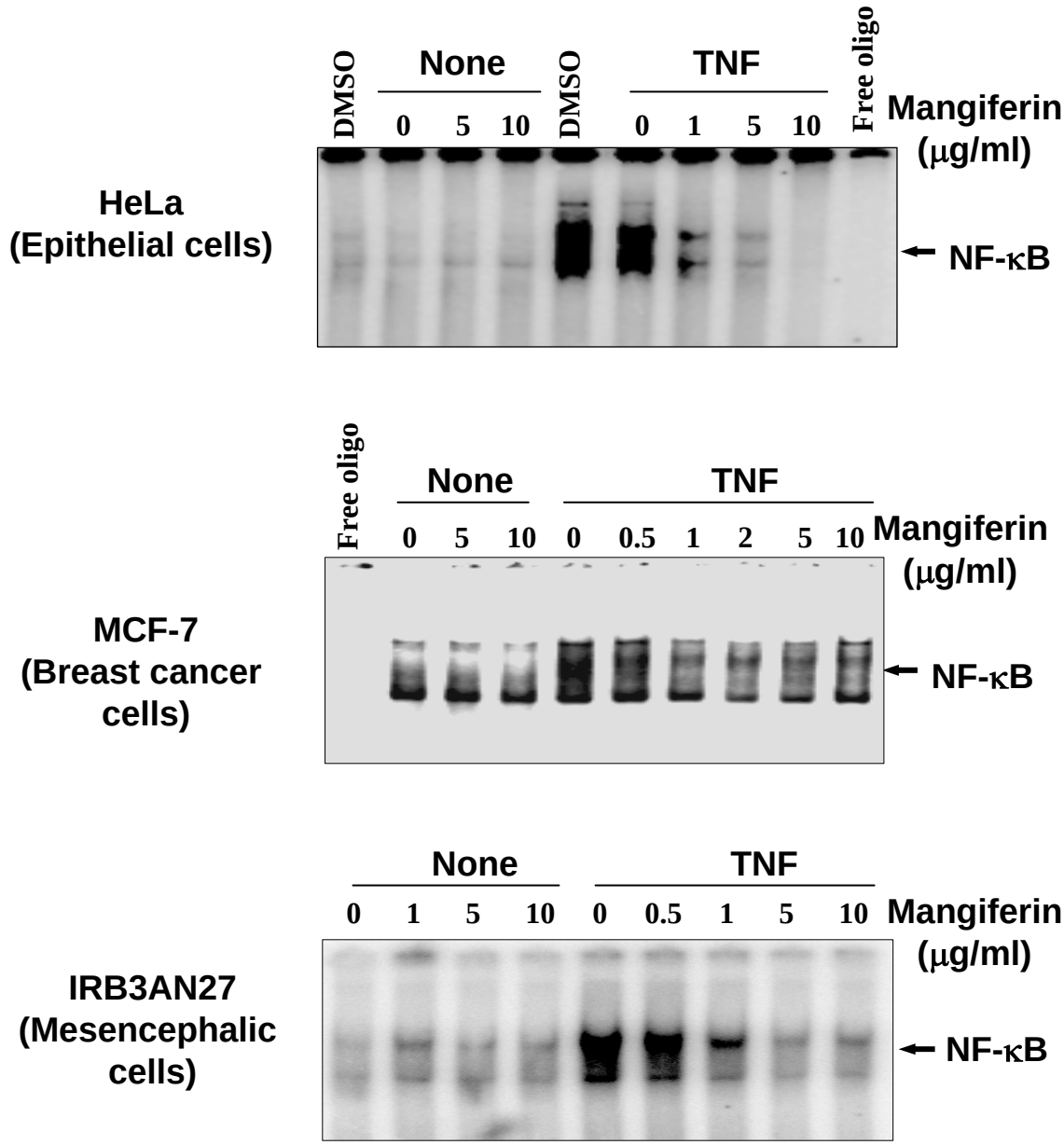


Fig.6.6. Effect of mangiferin on activation of NF-κB induced by TNF-α in different cell lines. HeLa (**A**), MCF-7 (**B**) and IRB3 AN27 (**C**) cells were incubated at 37°C with different concentrations of mangiferin for 3 h and then stimulated with 0.1 nM TNF for 30 min. After these treatments, nuclear extracts were prepared and assayed for NF-κB.

epithelial (HeLa), MCF-7 (breast cancer cells) and IRB3 AN27 (mesencephalic) cells (Fig.6.6). An almost complete inhibition in all the cell types suggests that this effect of mangiferin is not restricted to U-937 cell line.

Delineating the site of action of Mangiferin in TNF signal transduction pathway.

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor (TNFR) with associated death domain (TRADD)**,** TRAF2, NIK followed by phosphorylation and subsequent degradation of IκBα (Nasuhara *et al.,* 1999; Karin M, 1998b). To delineate the site of action of mangiferin in the TNF-signaling pathway leading to NF-κB activation, cells were transfected with TNFR1, TRADD, TRAF2, NIK, IKK and p65 plasmids, and then NF-κB dependent SEAP expression was monitored in untreated and mangiferin-treated cells. As shown in Figure 6.7.1, mangiferin suppressed TNFR1, TRADD, TRAF2, NIK, IKK induced gene expression but had little effect on p65-induced NF-κB reporter expression. The significant levels of p65 and TRAF2 in the transfected cells with p65 and TRAF2 plasmids showed the expression of the proteins by Western blot analysis (Fig.6.7.2). The specificity of the assay results is also demonstrated by the suppression of TNF- induced NF-κB reporter activity by dominant-negative IκBα plasmid. Thus, mangiferin must act at a step downstream from IKK and upstream of p65.

Mangiferin blocks TNF-induced ROI generation.

Previous reports have shown that one of the ways by which TNF activates NF-κB is through generation of ROI (Manna *et al.,* 1998; Manna *et al., 1999;* Li and Karin, 1999., Kumar and Aggarwal, 1999; Bowie *et al.,* 1997). Since mangiferin inhibits TNF mediated activation of NF-κB, we were interested to know the effect of mangiferin on the generation of ROI. This was examined by flow cytometry. U-937 cells were pretreated with different concentrations of mangiferin (1-10 μg/ml) for 3 h and then stimulated with TNF for 1 h. As indicated in Figure 6.8.1, TNF activated ROI which was inhibited by mangiferin in a dose depended fashion. We compared our results with

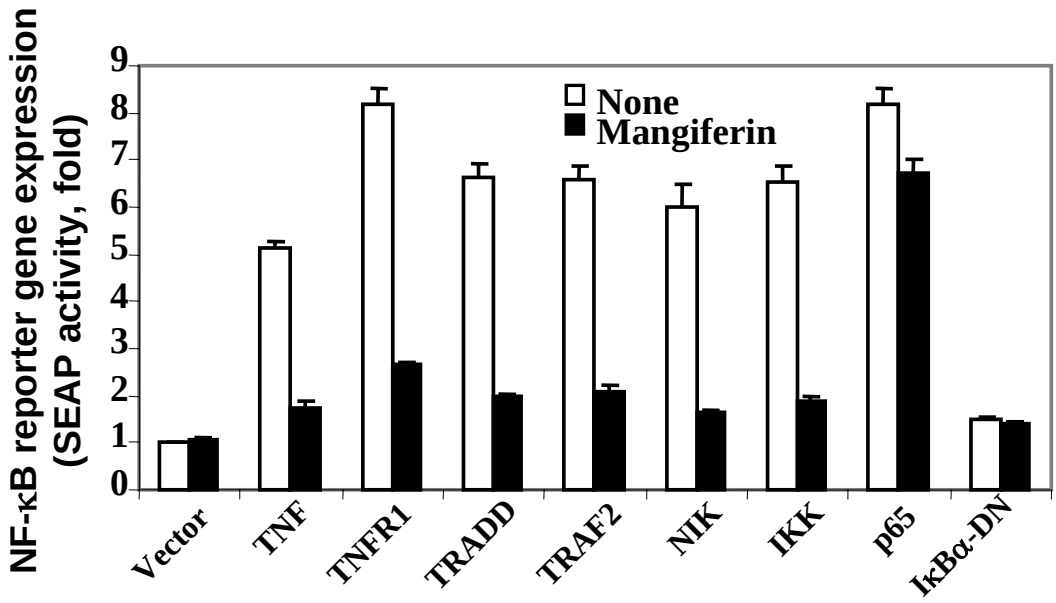


Fig.6.7.1. Mangiferin inhibits TNFR1-, TRADD-, TRAF-2-, NIK- and IKK-mediated but not p65-mediated NF-κB-dependent reporter gene expression. U-937 cells were either untreated or treated with mangiferin (10 μg/ml) for 3 h and then transiently transfected with the indicated plasmids along with an NF-κB responsive promoter linked to the SEAP gene and cultured for 12 h. Cells were assayed for SEAP activity as described in Experimental Procedures. Results are expressed as fold activity over the vector-transfected control

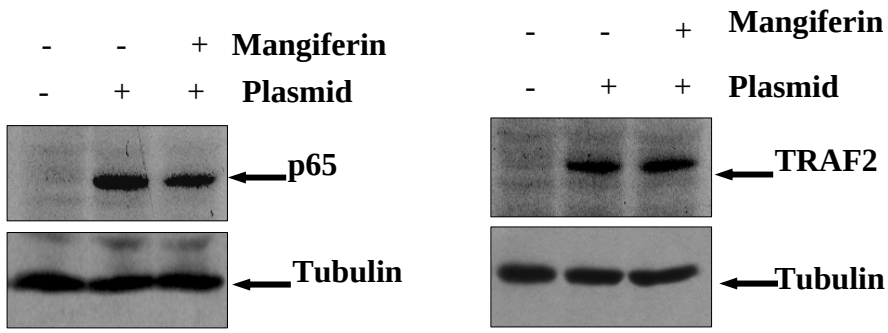


Fig.6.7.2. Demonstration of equal expression of plasmids transfected. As expression control, extracts from p65 and TRAF2 plasmids transfected cells were used to detect p65 and TRAF2 by Western blot.

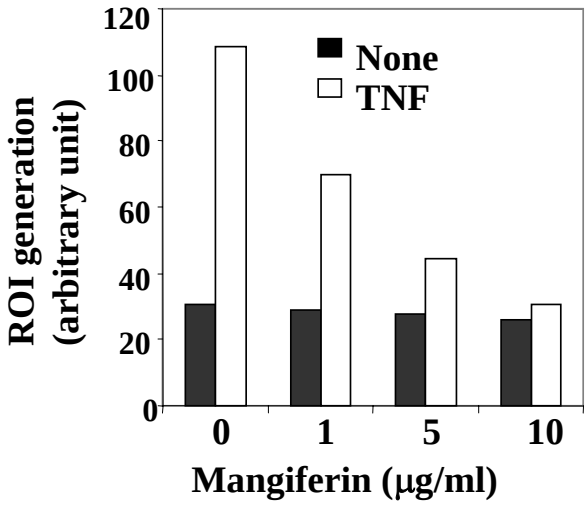


Fig.6.8.1 Effect of mangiferin on the levels of ROI. U-937 (3 $\times 10^6$ cells) were treated with different concentrations of mangiferin for 3 h then treated with and without TNF (0.1 nM) for 1 h. The level of ROI was measured as described in materials and methods.

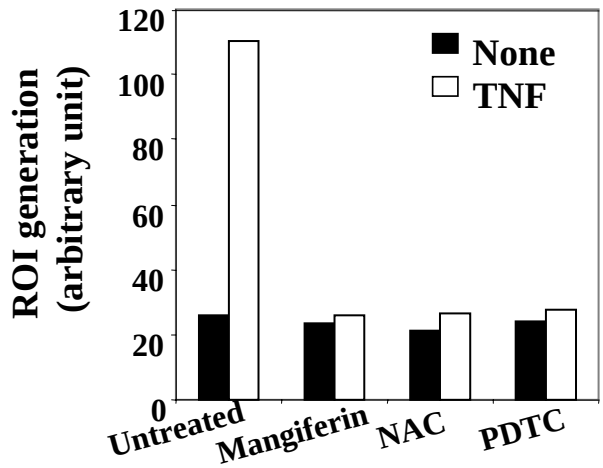


Fig.6.8.2. The levels of ROI generation by mangiferin and other antioxidants. Cells were treated with mangiferin, PDTC, and NAC for 3 h and then stimulated with TNF (0.1 nM) for 1 h. Then ROI was measured as described in materials and methods.

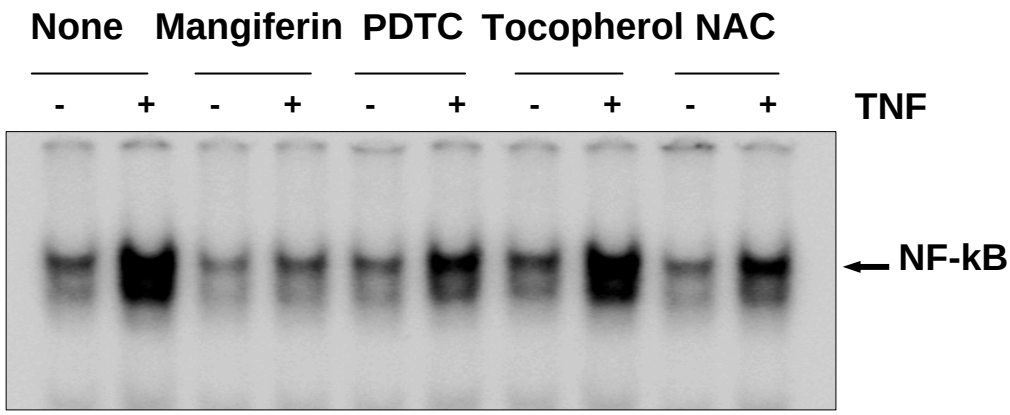


Fig. 6.8.3. Effect of different anti-oxidants on TNF-α induced NF-κB activation. Cells, treated with PDTC (100 μM), NAC (5 μM), tocopherol (100 μM) and Mangiferin (10 μg/ml) for 3 h were stimulated with or without TNF (0.1 nM) and the nuclear extracts were then assayed for NF-κB.

NAC, PDTC, and tocopherol, which are known antioxidants and modulate NF-κB activity. Our results show that mangiferin, NAC, PDTC, and tocopherol all inhibited TNF mediated NF-κB acivation (Fig 6.8.3). Also mangiferin like that of NAC and PDTC inhibits TNF activated ROI generation (Fig. 6.8.2). Thus, it is likely that mangiferin blocks TNF signaling through suppression of reactive oxygen intermediate generation.

Mangiferin modulates intracellular thiol level.

Reports have shown that the replenishment of intracellular cysteine which is required to produce reduced glutathione (GSH), the major intracellular thiol by NAC regulates NF-κB (Bowie *et al.,* 1997; Meyer *et al.,* 1994; Rahman and Mac Nee, 2000). As glutathione plays an important role in maintaining the redox status of the cell, we wanted to assay the levels of glutathione in mangiferin-pretreated cells. TNF treatment for 1 h was found to activate GSH. Mangiferin pretreatment led to increase in GSH level in a dose dependent manner. However there was no significant change in GSH levels in TNF treated cells (Fig 6.8.4 A). But, surprisingly, the level of Glutathione in mangiferin-pretreated cells was higher than that of NAC and PDTC pretreated cells (Fig 6.8.4 B). Since the ratio of GSH/GSSH determines the redox status of a cell, we also measured the levels of GSH and GSSH in cells treated with mangiferin and NAC separately. As shown in Figure 6.8.5 A and 6.8.5 B, cells pretreated with mangiferin show higher GSH and lower GSSH levels respectively, when compared to the untreated or even the NAC pretreated cells. Thus, by increasing the GSH/GSSG ratio more than the known anti-oxidants like NAC, Mangiferin seems to be a much more potent anti-oxidant.

Increase in catalase activity by mangiferin.

Catalase is one of the antioxidant enzymes whose overexpression is reported to downregulate NF-κB levels (Imbert *et al.,* 1996). Since mangiferin inhibited NF-κB and

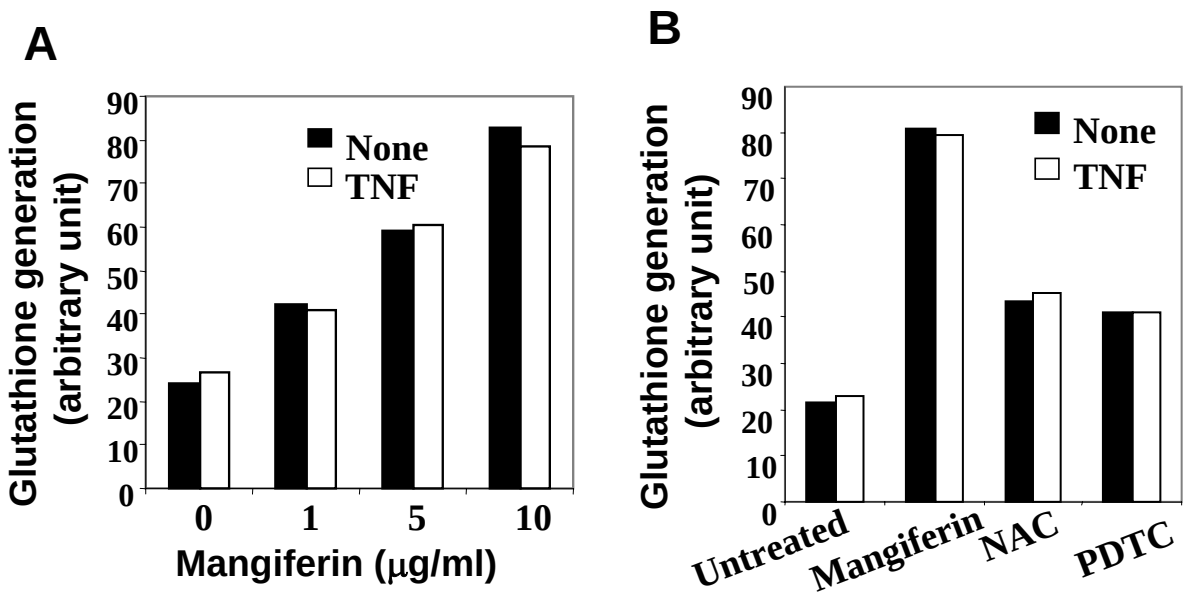


Fig.6.8.4 Effect of mangiferin on the levels of Glutathione. A. U-937 (3×10^6 cells) were treated with different concentrations of mangiferin for 3 h then treated with and without TNF (0.1 nM) for 1 h. **B.** Cells were treated with mangiferin, PDTC, and NAC for 3 h and then stimulated with TNF (0.1 nM) for 1 h. The level of glutathione was measured as described in materials and methods.

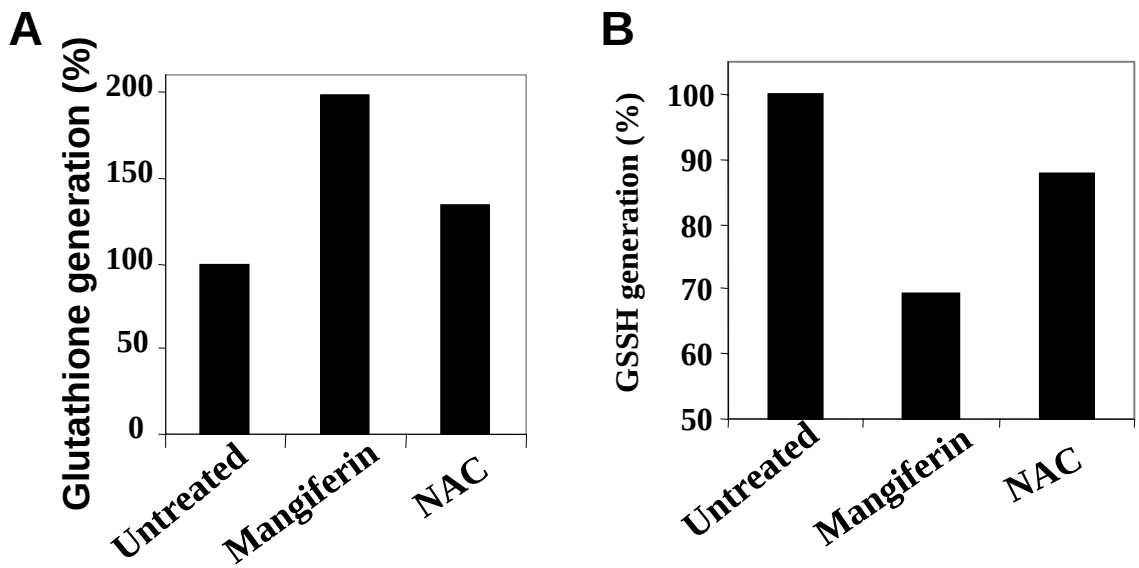


Fig.6.8.5. Comparison of the levels of GSH and GSSG generation by mangiferin and NAC. Cells were treated with mangiferin (10 μg/ml) and NAC (5 μM) for 3 h and measured for GSH (**A**) and GSSG (**B**) as described in materials and methods.

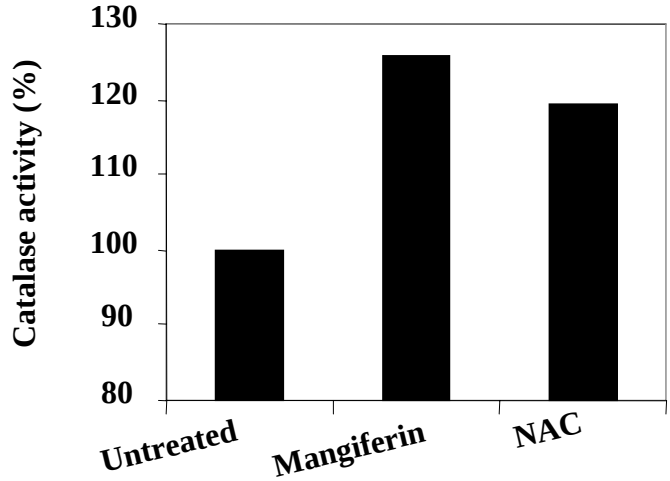


Fig.6.8.6. Comparison of the levels of Catalase generation by mangiferin and NAC. Cells were treated with mangiferin (10 μg/ml) and NAC (5 μM) for 3 h and measured for catalase as described in materials and methods.

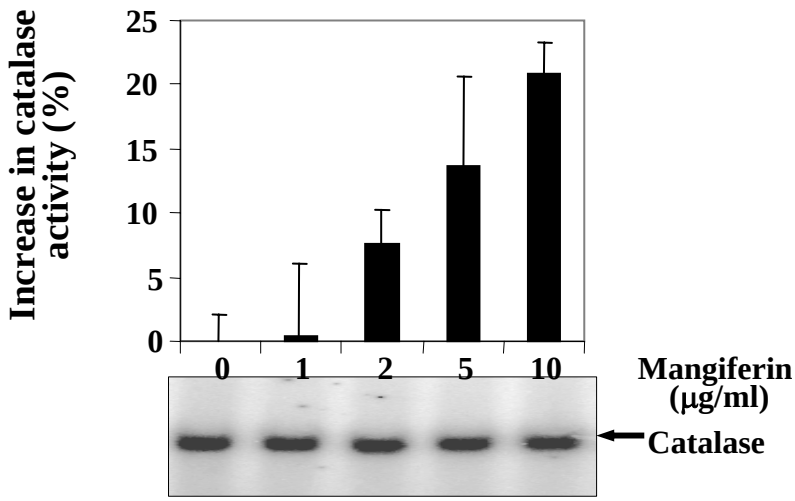


Fig.6.8.7. Effect of mangiferin on catalase activity. Cells treated with different concentrations of mangiferin for 3 h and the catalase activity was assayed. 100 μg of extract proteins were analyzed in 8.5% SDS-PAGE and catalase was detected using anti-catalase monoclonal antibody by Western blot.

showed antioxidant activities, we were interested to check the catalase activity also. Mangiferin was found to show a marginal increase (23%) in the catalase activity compared to untreated cells (Fig. 6.8.6). Mangiferin showed a dose-dependent increase in catalase activity without changing the level of protein (Fig.6.8.7). To detect the role of GSH level and catalase activity in mangiferin-mediated response, we pretreated the cells with BSO, inhibitor of GSH biosynthesis or ATZ, irreversible inhibitor of catalase and then treated with 10 μg/ml mangiferin for 3 h. Cells were then incubated with TNF (0.1 nM) for 1 h and then GSH level or catalase activity was assayed from whole cell extract and NF-κB were assayed from nuclear extract of similarly treated cells. Mangiferin alone or in combination with TNF increased GSH level almost 4-fold and BSO treatment inhibited this to a significant extent (Fig 6.9.1). Mangiferin alone or in combination with TNF increased catalase activity by 22% and 20% respectively. In ATZ-pretreated cells mangiferin or in combination with TNF did not activate catalase activity (Fig.6.9.2). Mangiferin inhibited TNF-induced NF-κB activation and BSO pretreated cells showed almost 80% protection of mangiferin-mediated downregulation of NF-κB. However, inhibition of catalase by ATZ did not protect mangiferin-mediated NF-κB inhibition significantly (Fig 6.9.3). These data suggest the potential involvement of GSH, but not a significant role of catalase in mangiferin-mediated downregulation of NF-κB.

Mangiferin induces GSH level in γ-GCS overexpressed cells.

γ-GCS is a rate-limiting enzyme in the synthesis of GSH. Since mangiferin increases the GSH levels, we examined the effect of mangiferin in the presence of γ-GCS. For this, cells with or without overexpressed γ-GCS were treated with 10 μg/ml mangiferin and then stimulated with different concentrations of TNF and the levels of NF-κB, ROI and GSH were measured. As γ-GCS is involved in synthesis of GSH, the cellular anti-oxidant, the cells overexpressing γ-GCS had basal levels of NF-κB and ROI but higher GSH. Mangiferin pretreated cells as expected inhibited the TNF-

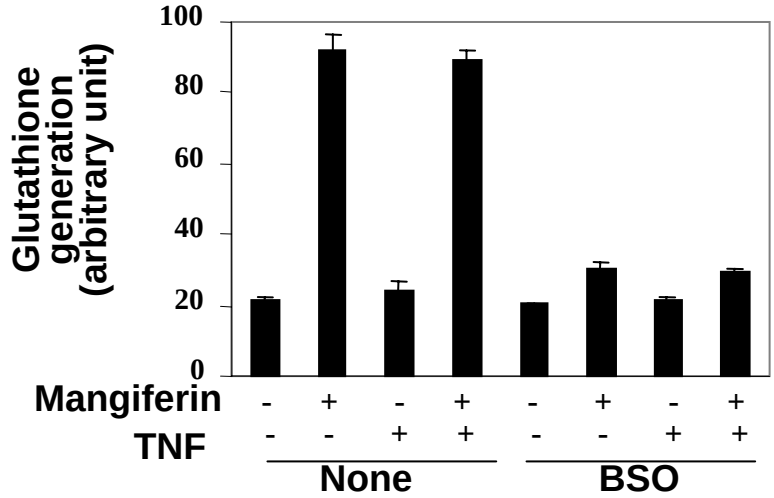


Fig. 6.9.1. Effect of BSO on mangiferin-mediated induction of GSH level activity. Cells were treated with BSO (0.5 mM) for 3 h and then treated with 10 μg/ml mangiferin for 3 h. Cells were then stimulated with 0.1 nM TNF for 1 h and level of GSH activity were measured as described in materials and methods.

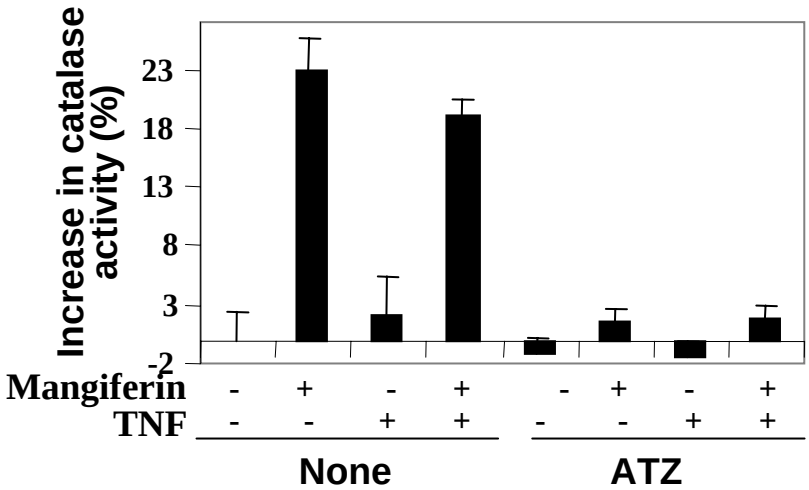


Fig.6.9.2. Effect of ATZ on mangiferin-mediated induction of catalase activity. Cells were treated with ATZ (1 mM) for 3 h and then treated with 10 μg/ml mangiferin for 3 h. Cells were then stimulated with 0.1 nM TNF for 1 h and level of catalase activity were measured as described in materials and methods.

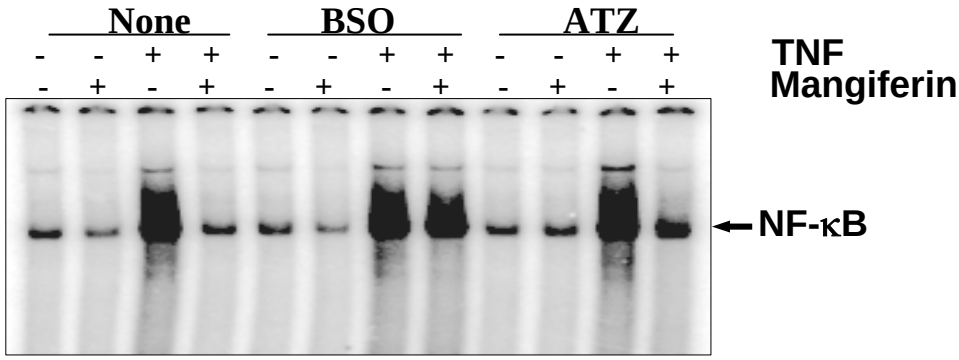


Fig.6.9.3. Effect of BSO and ATZ on mangiferin-mediated inhibition of NF-κB. Cells were treated with BSO (0.5 mM) or ATZ (1 mM) for 3 h and then treated with 10 μg/ml mangiferin for 3 h. Cells were then stimulated with 0.1 nM TNF for 1 h and NF-κB was assayed from nuclear extract.

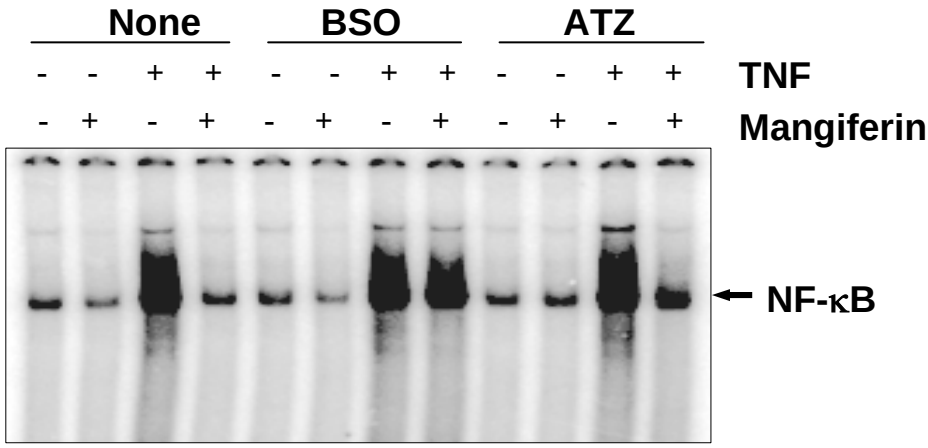


Fig.6.9.3. Effect of BSO and ATZ on mangiferin-mediated inhibition of NF-κB. Cells were treated with BSO (0.5 mM) or ATZ (1 mM) for 3 h and then treated with 10 μg/ml mangiferin for 3 h. Cells were then stimulated with 0.1 nM TNF for 1 h and NF-κB was assayed from nuclear extract.

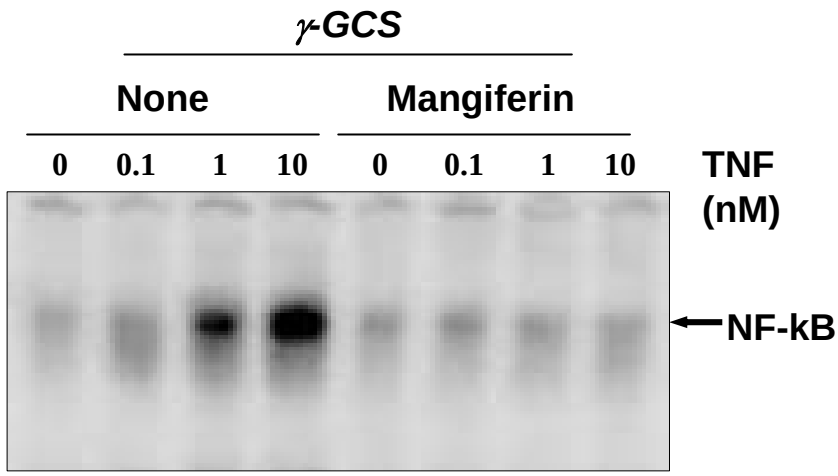


Fig.6.10.1. Effect of mangiferin on NF-κB activation in γ-GCS overexpressed cell line. Rat hepatoma cells, stably transfected with either vector (*neo*) or gamma glutamylcysteine synthetase *(γ-GCS*) were treated with mangiferin (10 μg/ml) for 3 h and then stimulated with 0.1 nM TNF for different times. Nuclear extracts were prepared and assayed for NF-κB.

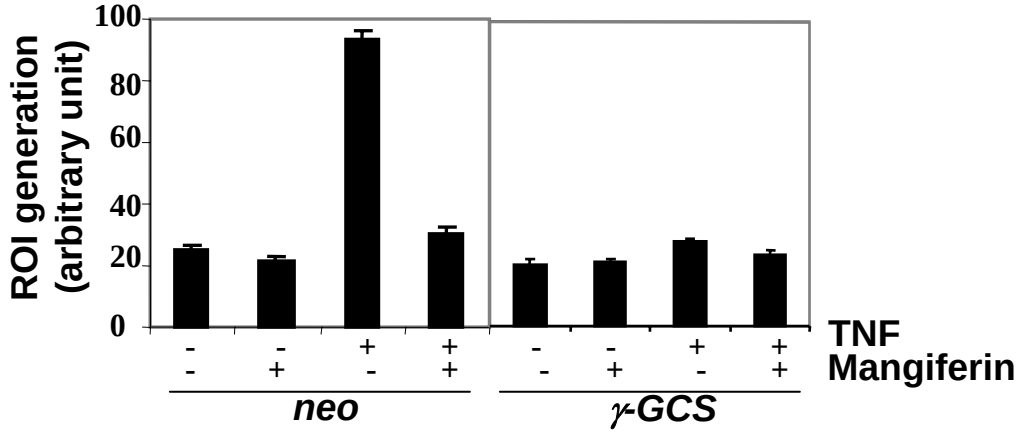


Fig.6.10.2. Effect of mangiferin on ROI production in γ-GCS overexpressed cell line. Rat hepatoma cells, stably transfected with either vector (*neo*) or gamma glutamylcysteine synthetase *(γ-GCS*) were treated with mangiferin (10 μg/ml) for 3 h and then stimulated with 0.1 nM TNF for different times. ROI liberated was measured as described in materials and methods.

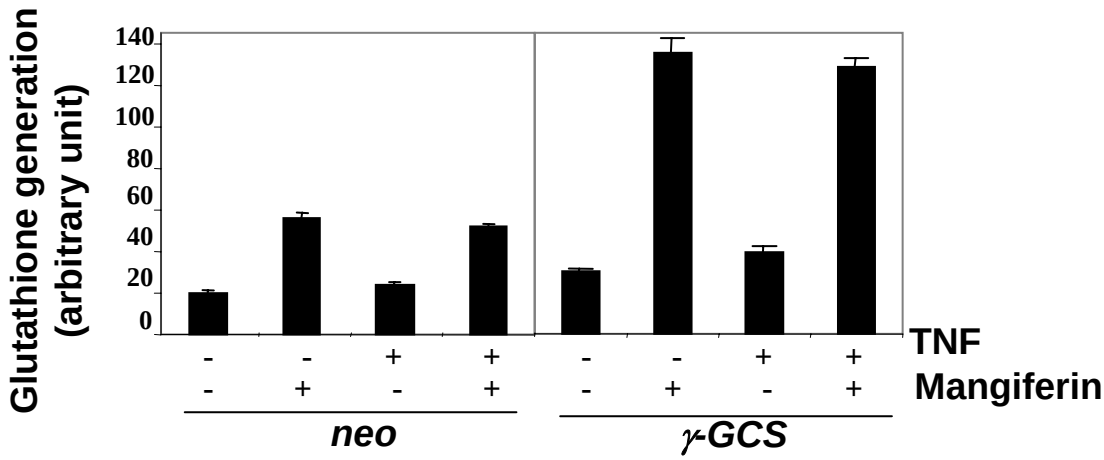


Fig.6.10.3. Effect of mangiferin on GSH amounts in γ-GCS overexpressed cell line. Rat hepatoma cells, stably transfected with either vector (*neo*) or gamma glutamylcysteine synthetase *(γ-GCS*) were treated with mangiferin (10 μg/ml) for 3 h and then stimulated with 0.1 nM TNF for different times. Cell extracts were prepared and assayed for GSH as described in materials and methods.

induced NF-κB activation (6.10.1) and ROI generation in vector control (*neo*) cells. Moreover it enhanced the ROI quenching (6.10.2) and GSH levels (6.10.3) in the γ-GCS overexpressed cells. From this we suspect that mangiferin might upregulate γ-GCS within the cells and thus enhance the GSH levels.

Mangiferin potentiates Cisplatin, Vincristin, Doxorubicin, Etoposide, Adriamycin and AraC mediated cell death.

NF-κB is known to be involved in apoptosis and since mangiferin was found to downregulate NF-κB, we hypothesized that mangiferin pretreatment might potentiate the apoptotic action of other apoptotic agents though by itself at the dose of 10 μg/ml mangiferin does not cause any significant cell death. U-937 cells were pre-incubated with 10 μg/ml mangiferin for 3 h followed by incubation for 72 h with or without different concentrations of TNF and checked for cell viability by MTT assay and also by fluoremetric Live & Dead cell assay. Results indicate that mangiferin on its own was not toxic (cell death was only 8%) to the cells but enhances the cell death mediated by TNF from 16% to 39% (Fig 6.11.1 and 6.11.2). To extend the study to other apoptotic agents, we pretreated U-937 cells with and without 10 μg/ml mangiferin for 3 h and then treated the cells with 1 μM of various apoptotic agents like cisplatin, vincristine, doxorubicin, etoposide, adriamycin, taxol, and AraC for 72h and then looked for the cell viability by MTT assay and fluorimetric Live & Dead cell assay. Mangiferin was again found to enhance the cell death mediated by these agents (Fig 6.11.3 & 6.11.4).

To prove that the potentiation of apoptosis is due to inhibition of NF-κB, we transiently transfected U-937 cells with *IκBα-DN* construct, which blocks the activation of NF-κB and *p65* construct and then looked for the cell death after 36 h by MTT and Live & Dead cell assay. The *IκBα-DN* transfected cells showed an increase in cell death by 12% alone and also potentiated cell death with TNF from 41% to 50%. Mangiferin increased the cell death mediated by TNF from 41 % to 53% and in *IκBα-DN* cells this cell death was further increased to 58%. In p65-overexpressed cells, the cell death was

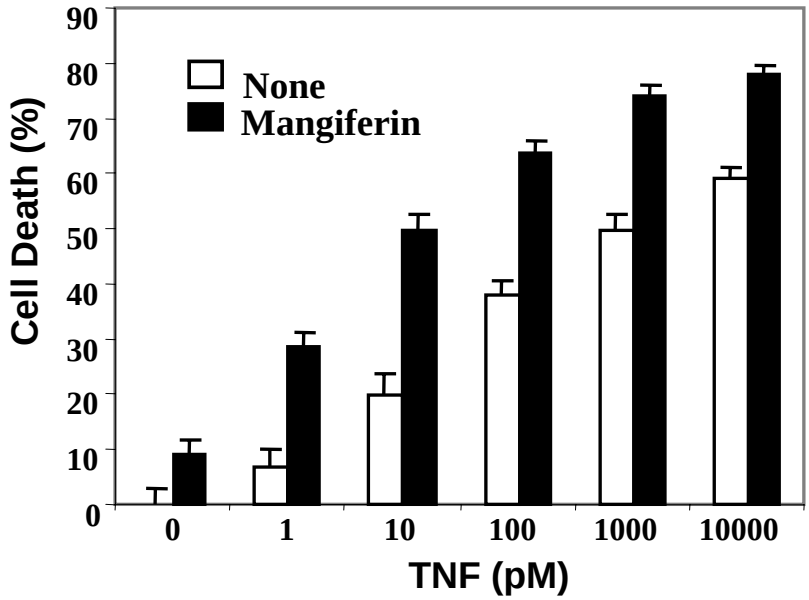


Fig.6.11.1. Effect of mangiferin on the cell death induced by TNF. U-937 cells were treated with or without 10 μg/ml mangiferin for 3 h and then treated with different concentrations of TNF and incubated at 37^0C for 72 h and the cell viability was assayed by MTT as described in materials and methods

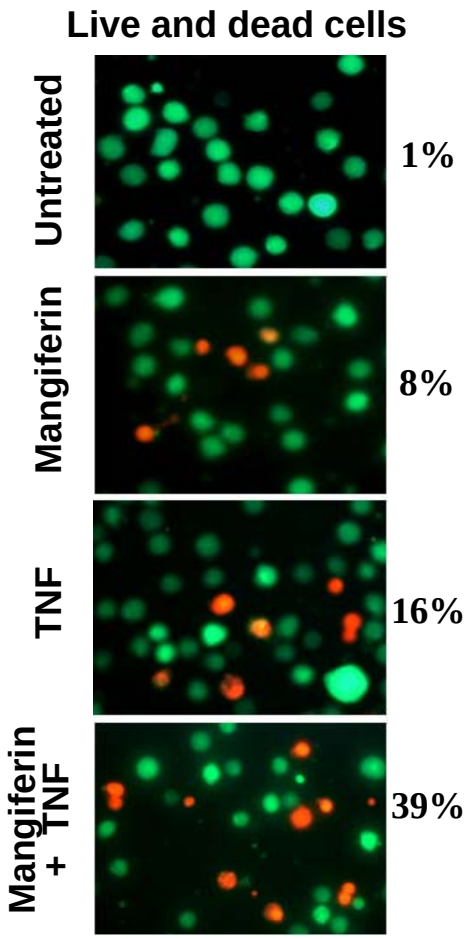


Fig.6.11.2. Effect of mangiferin on the cell death induced by TNF. Cells were treated with or without 10 μg/ml mangiferin for 3 h and then stimulated with 1 nM TNF for 24 h. The cell viability was assayed by Live and Dead cells assay as described in materials and methods.

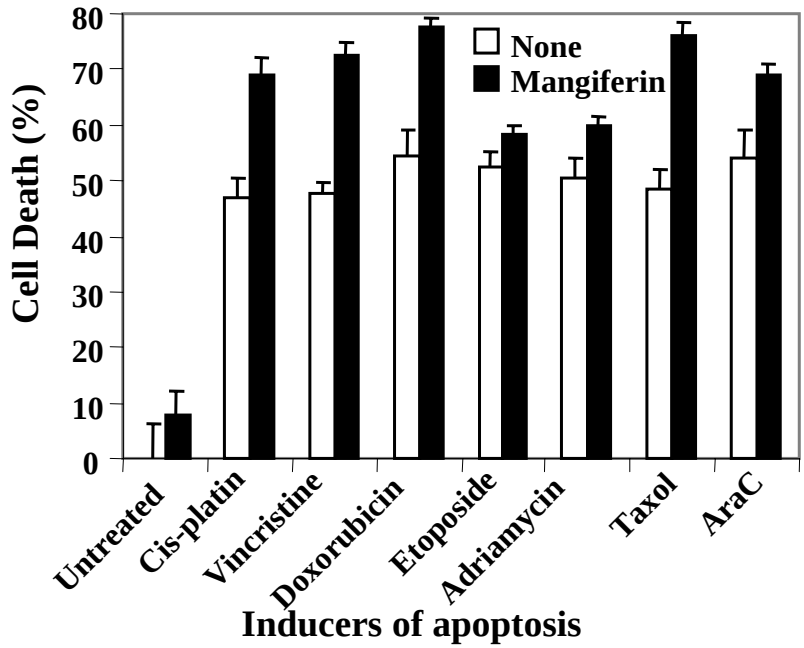


Fig.6.11.3. Effect of mangiferin on the cell death induced by various apoptotic agents. Cells were pretreated with and without mangiferin (10 μg/ml) for 3 h and then incubated with 1 mM of each of cis-platin, vincristine, doxorubicin, etoposide, adriamycin, taxol, and AraC for 72 h. Cell death was assayed by MTT assay.

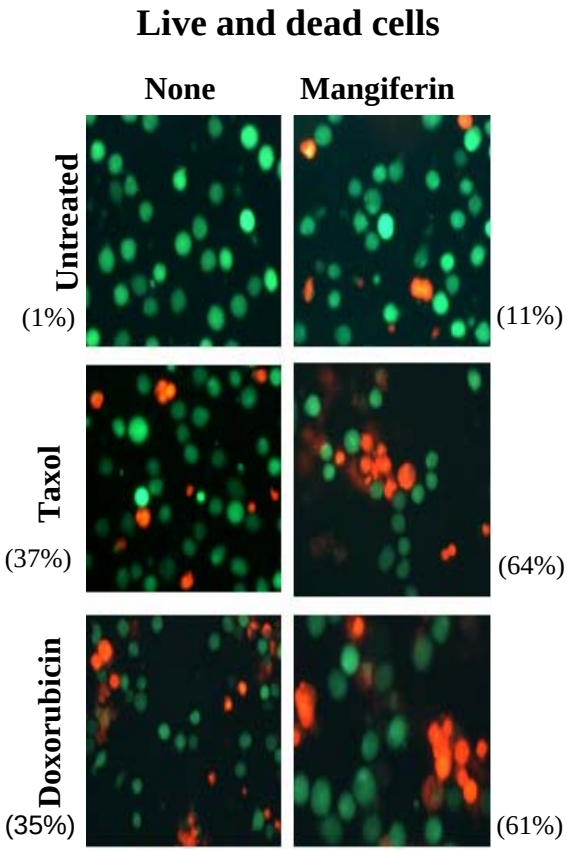


Fig.6.11.4. Effect of mangiferin on the cell death induced by various apoptotic agents. Cells were pretreated with 10 μg/ml mangiferin for 3 h and then stimulated with taxol (1 mM) and doxorubicin (1 mM) for 24 h. The cell viability was assayed by 'Live and Dead cell assay'

not observed when cells were treated with TNF or combination of TNF and mangiferin (Fig 6.11.5 & 6.11.7). The *IκBα-DN* transfected cells downregulated NF-κB and p65 transfected cells showed upregulation of NF-κB (Fig. 6.11.6). Overall our data clearly show the involvement of NF-κB in cell proliferation and inhibition of this leads of cell death. Downregulation of NF-κB sensitizes cells to chemotherapeutic agents mediated apoptosis. Our results thus indicate that mangiferin, like other anti-oxidants is not toxic to the cells but by inhibition of NF-κB, it can serve as potential drug in combination therapy with other well-known chemotherapeutic agents.

6.3 Discussion

Since mangiferin is known to elicit anti-inflammatory (Garcia *et al.,* 2002; Sanchez *et al.,* 2000), anti-carcinogenic, and anti-diabetic properties and all these events occur through regulation of NF-κB, we hypothesized that these effects are mediated through suppression of NF-κB activation, an early mediator of the pleiotropic effects of the inflammatory cytokine, TNF. Our results clearly demonstrate that mangiferin suppresses NF-κB activation induced by TNF and other inflammatory agents. The inhibition of NF-κB activation by mangiferin is correlated with the suppression of IκBα phosphorylation and degradation, p65 nuclear translocation, p65 phosphorylation and NF-κB dependent reporter gene transactivation. We also found that NF-κB regulated genes involved in inflammation COX2 and ICAM1 are downregulated by mangiferin.

There are various ways by which mangiferin might inhibit TNF induced NF-κB activation. This involves the sequential interaction of TNF receptor with TRADD, TRAF2, and NIK, which then activates IκBα kinase (IKK) and IKK in turn phosphorylates IκBα. NF-κB activation requires sequential phosphorylation, ubiquitination, and degradation of IκBα. Mangiferin blocks IKK activation followed by IκBα phosphorylation and degradation indicating that mangiferin's effect on NF-κB may be due to inhibition of phosphorylation and the proteolysis of IκBα. However our findings suggest that mangiferin blocks NF-κB dependent reporter gene expression

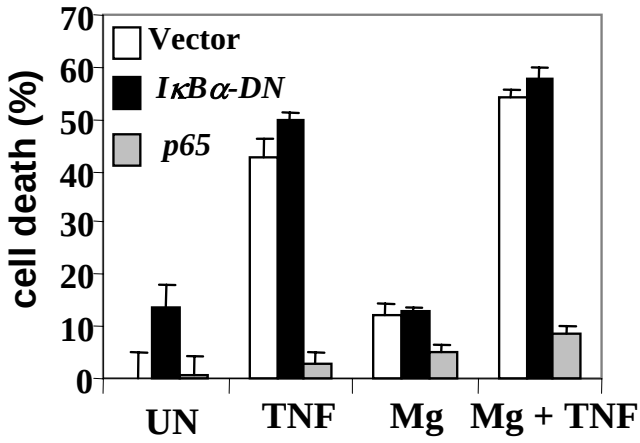


Fig.6.11.5. Involvement of p65 in cell death. U-937 cells were transiently cotransfected with *IκBα-DN* construct, *p65* construct for 12 h. Cells were then treated with 10 μg/ml mangiferin for 3 h and followed by stimulation TNF (1 nM) for 36 h. Cell viability was assayed by MTT.

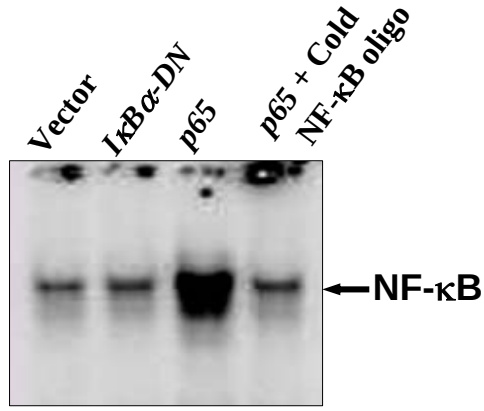


Fig.6.11.6. Involvement of p65 in cell death. U-937 cells were transiently cotransfected with *IκBα-DN* construct, *p65* construct for 12 h. Cells were then treated with 10 μg/ml mangiferin for 3 h and followed by stimulation TNF (1 nM) for 36 h. nuclear extract was used to assay NF-κB.

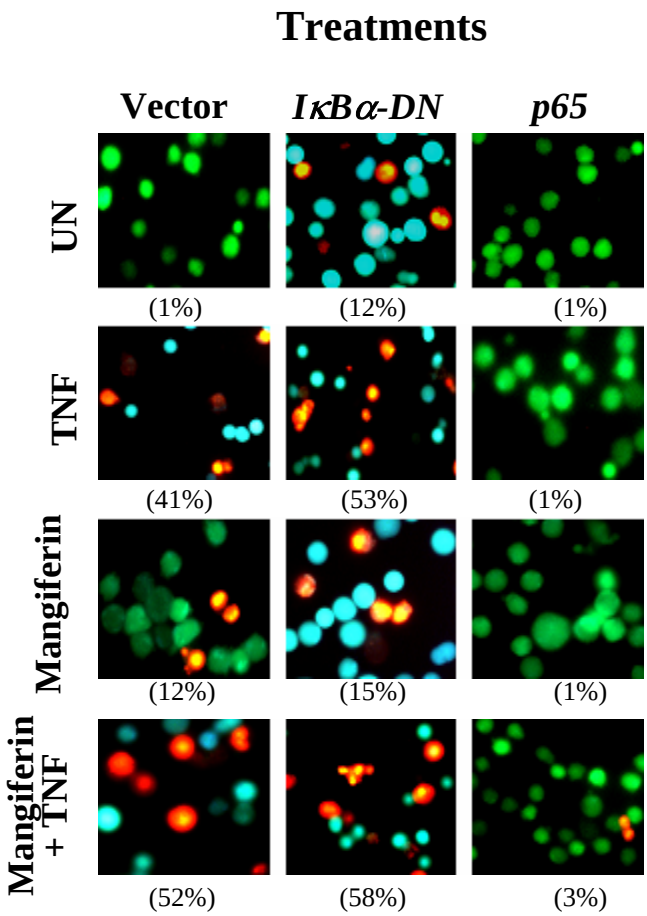


Fig.6.11.7. Involvement of p65 in cell death. U-937 cells were transiently cotransfected with *IκBα-DN* construct, *p65* construct for 12 h. Cells were then treated with 10 μg/ml mangiferin for 3 h and followed by stimulation TNF (1 nM) for 36 h. Cell viability Live & Dead cell assay as described in materials and methods.

induced by TNFR1, TRADD, TRAF2, NIK and IKK but not by p65, suggest that mangiferin is acting by some other mechanism. Our results also indicate that mangiferin may be inhibiting these kinases through an anti-oxidant mechanism.

We have found that mangiferin suppresses NF-κB activation induced by wide variety of agents, including TNF, ceramide, SA-LPS and PMA, in U-937 cells. These results indicate that it is a broad-spectrum inhibitor of NF-κB and it is also not cell type specific as the inhibition was noticed in human cells like U-937 (human histiocytic lymphoma), HeLa (epithelial cells), MCF-7 (breast cancer cells), and also in murine IRB3 AN27 (mesencephalic) cells.

Our results show that mangiferin inhibits ROI generation in U-937 cells. Thus, it is possible that the effects are mediated through quenching of reactive oxygen intermediates. GSH is an important cellular reductant involved in detoxification of ROI, and we observed an increase in the GSH level by mangiferin treatment. Since the GSH/GSSG ratio is fundamental to the transcriptional activation of several pro-inflammatory and antioxidant- protective genes we also measured the GSSG levels in the same extracts. We found a subsequent decrease in the GSSG upon mangiferin pretreatment (Fig. 6.8.5). We compared our results with NAC and PDTC which are free radical scavengers, which increase intracellular GSH levels, and are known to inhibit TNF induced activation of NF-κB (Manna *et al.,* 1998; Manna *et al.,* 1999; Futakuchi *et al.,* 1998). Mangiferin was found to increase the GSH/GSSG ratio more than even NAC. Thus mangiferin is also probably acting by increasing GSH level and thereby downregulating NF-κB and its downstream effects. As one of the mechanism of TNF mediated NF-κB activation, involves through ROI generation, mangiferin could inhibit TNF mediated action by quenching ROI generation. In this process it activates the intracellular redox sensing GSH. The inhibition of GSH biosynthesis using BSO mimicked the mangiferin-mediated downregulation of NF-kB indicating involvement of GSH in mangiferin-mediated response (Fig. 6.9.1 & 6.9.3). As biosynthesis of GSH involves the activation of γ-GCS, the rate limiting enzyme we found an increase in the level of GSH upon mangiferin treatment in *γ-GCS* overexpressed cells from 3-fold to

6.5 fold (Fig.6.10.3). We therefore speculate that the mangiferin-mediated increase in GSH level is due to activation of γ-GCS. Although mangiferin showed a marginal increase in the activity of catalase, an antioxidant enzyme but inhibition of catalase did not mimic the mangiferin-mediated downregulation of NF-κB.

As chemotherapeutic agent-induced resistance is the main problem for cancer, the understanding of the basic mechanism will be helpful in addressing this problem. There was almost 20–40 % survival in cisplatin-, vincristine-, doxorubicin-, etoposide-, adriamycin-, Taxol-, araC- treated cells. These agents showed potentiation of cell killing in combination with mangiferin (Fig.6.11.3.). As *IκBα-DN* transfected cells, which showed downregulation of NF-κB (Fig.6.11.6) potentiated cell death mediated by TNF (Fig.6.11.7), it proves that the downregulation of NF-κB is responsible for potentiation of apoptosis.

Our results bring into light the mechanism of action of a new plant polyphenolic compound mangiferin, whose suppressive effects on NF-κB, with increase in intracellular GSH levels, may prove beneficial as an anti-inflammatory, antidiabetic, and anticarcinogenic drug. And, with the ability to potentiate the cell death induced by other chemotherapeutic agents, mangiferin may also serve as an effective drug in combination therapy.

DISCUSSION

Human beings or animals must defend themselves against multitude of different pathogens including bacteria, viruses, fungi, and protozoan/metazoan parasites as well as tumors and a number of other harmful agents. In order to respond to such wide range of antigens, our body has developed a complex mechanism broadly termed as ***INFLAMMATION.*** Inflammation has thus been one of the earliest addressed problems of biology. The ultimate aim of inflammation is to restore the inflamed or damaged tissue to its original state. In this process, cells of our body liberate various cytokines and chemokines such as, TNF, IL-1, IL-6, IL-8 and reactive oxygen species, which are help to combat the foreign agents. However, an aberrant inflammatory response is equally deleterious as evidenced by a range of disorders where excessive inflammation is the cause. Some of these include various forms of allergies and arthritis, asthma, sepsis, cardiovascular diseases, neurodegenerative disorders and also cancer. To develop effective drugs to treat these life threatening diseases, one needs to thoroughly understand the functioning of the cells of immune system.

The network of signals in the cell has check-points that are of greatest importance for the understanding of intra-cellular and inter-cellular regulation. From the biomedical point of view they represent the future targets of pharmacological interventions. Nuclear factor κB (NF-κB) signaling belongs to these crossroads. NF-κB is a transcription factor belonging to a family (REL family) of structurally related eukaryotic transcription factors that promote the expression of genes involved in a variety of cellular processes including cell growth, proliferation and cell death. The members of the family form homo and hetero dimers, and the most common active form being the heterodimer of p50 and p65 (Rel A), which bind to a sequence motif known as the κB site found in the promoter region of various genes and thus control their expression. While needed for the proper functioning of the immune system, inappropriate activation of NF-κB can mediate inflammation and tumorigenesis. Most of the molecules (cytokines, chemokines, growth factors, adhesion molecules etc) involved in inflammation and tumor progression have in their promoter region κB sites responsive to NF-κB. Knocking out of NF-κB subunits is however not a

viable strategy due to its significance in normal functioning of the cell. Hence the better way is to inhibit the abberant activation of this transcription factor.

Several drugs are available in the market, which are being successfully employed in the treatment of several inflammatory disorders and cancer. However they suffer from bringing with them side effects and resistance over the period of treatment. Hence there is an increasing demand for drugs that can ameliorate a patient's distress and is also less toxic. In such a scenario, drugs based on the body's endogenous resoluters such as some prostacyclins, glucocorticoids, annexins and resolvins, seems to be promising. Research over the last two decades has brought to light another important endogenous resoluter, the alpha- melanocyte stimulating hormone. Alternative to these endogenous resoluters, another class of drugs which are gaining importance is plant based products. Of the therapeutic drugs in use today, 70% of them are plant derived. The main reason for the acceptance of such natural resoluters or plant products is their effective role in treatment of inflammation or cancer without the side effects. However, before these drugs are brought to the market, one need to thoroughly understand their mechanism of action to make best use of them.

Hence, in the present work, we aimed to understand the molecular mechanism of action of two such putative drugs– One being α-MSH and the other, a plant polyphenol, Mangiferin.

Research over the last two decades has established a close link between the neuroendocrine system and the immune system. An ideal link in this system is the α-melanocyte stimulating hormone (α-MSH). It is a highly evolutionarily conserved neuropeptide released from the pituitary gland and well known for its role in melanogenesis. The level of α-MSH was found to be high in some inflammatory diseases as compared to the normal patients. This led to explore the possible role of α-MSH in these diseases. Receptors of α-MSH (melanocortin receptors) were detected on several cells of the body including monocytes, macrophages, leukocytes, and epithelial cells indicating α-MSH does more than just pigment regulation and indeed research in the last decade has shown that α-MSH possesses remarkable anti-

inflammatory and anti-pyretic property and thus could fall under the category of endogenous pro-resoluters. It exerts its function through a family of G-protein coupled melanocortin receptors (MC-1R to MC-5R). However, the molecular mechanism of action of this hormone is yet not clearly understood. We therefore aimed to understand the mechanism (s) of action of α-MSH in some of the important cells of the immune system.

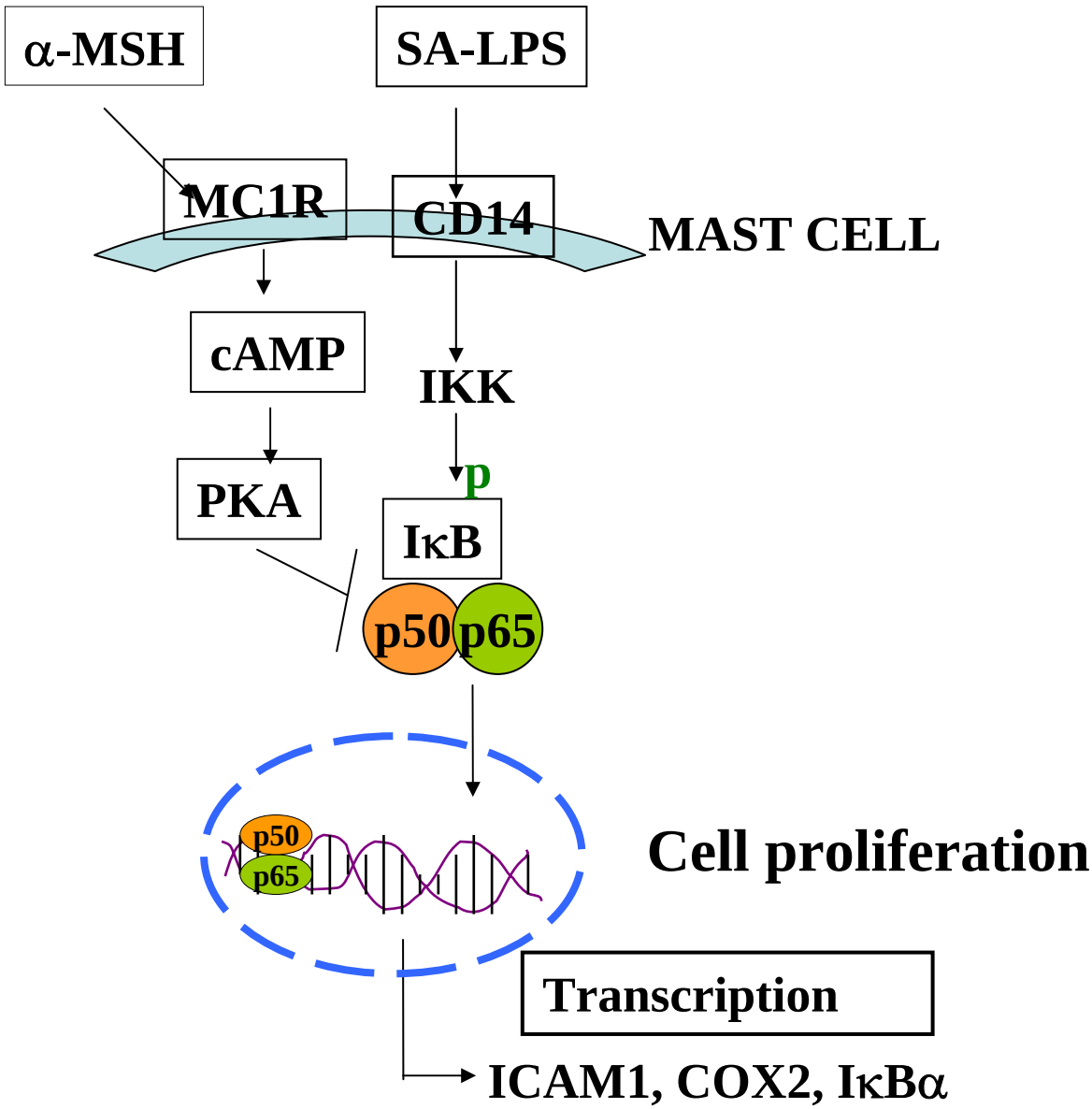
To begin with we examined the effect of α-MSH on mast cells, which are the key responders to allergic and inflammatory disorders. Murine mast cell line, MC-9 which was positive for MC-1R (Fig. 3.2.2), was used in most of our work. α-MSH was found to inhibit endotoxin (serum activated-lipopolysacharide, SA-LPS, 100 ng/ml) mediated NF-κB activation in a time (24 h onwards) and dose (10 nM onwards) dependent manner. It also inhibited NF-κB dependent reporter gene (heat stable secretory alkaline phosphatase, SEAP) and ICAM1 expression (Fig. 3.1). Since NF-κB is also an important cell survival factor, we checked if prolonged treatment of α-MSH affected the cell viability of MC-9. Treatment of α-MSH beyond 36 h resulted in decrease in cell viability as measured from MTT assay (Fig. 3.3.1), ^{3}H Thymidine incorporation (Fig. 3.3.2), and increase in ROI generation and lipid peroxidation (Fig. 3.3.3). Cell death was further confirmed with markers of apoptosis, caspase-8 activation and PARP cleavage. However, to our surprise, we did not observe any significant amount of cell death in a monocytic cell line, U-937. The cell death observed was confirmed to be due to inhibition of NF-κB as, the IκB-α DN transfected MC-9 or p65 over expressing Hut 78 cell line, protected against α-MSH mediated cell death (Fig. 3.6). α-MSH mediated NF-κB down regulation occurred via activation of adenylate cyclase, followed by generation of cAMP and activation of PKA since inhibitors of any of the above led to reversal of α-MSH mediated actions (Fig. 3.5). The results were reproducible in human mast cell line HMC-1 and mouse derived mast cells also. As mast cells are key players of allergic and inflammatory reactions such as asthma, arthritis, the regulation of their number by α-MSH might prove to be beneficial.

Next we studied the effect of α-MSH on macrophages, which constitute the first line of body's defense system. They respond to microbial pathogen by recognizing bacterial endotoxin (LPS) mainly via the CD14 (endotoxin receptor) expressed on their surface. We used for our study THP-1 derived macrophages that were found to express CD14. Pretreatment of cells with α-MSH (10 nM) led to significant reduction in the enzymes released upon stimulation with endotoxin (SA-LPS, 100 ng/ml) such as myeloperoxidase and β-D-glucoronidase. α-MSH also inhibited oxidative burst response, Nitric oxide (NO) generated and ROI produced in response to endotoxin. We also observed inhibition of endotoxin-activated NF-κB and its responsive gene ICAM1 in presence of α-MSH. There are reports showing downregulation of CD14 to be one of the mechanisms involved in regulation of LPS mediated responses (Nemoto E, 2000). Our results also indicated that α-MSH led to downregulation of CD14, from cell surface. Unlike the work by Vega *et.al.,* where they showed that geldamycin downregulates CD14 from cell surface and accumulates it in the endoplasmic reticulum, we found that a-MSH release the CD14 into the culture supernatant (Fig. 4.5). Pretreatment with anti-MC1R antibody reversed the effect of α-MSH. The results thus indicate that α-MSH acts via its receptor MC-1R to stimulate macrophages to release the CD14 from cell surface and thereby down regulates SA-LPS mediated biological activities. Our data highlights the potential role of α-MSH in treatment of diseases like sepsis where endotoxin mediated activation of macrophages is an important cause.

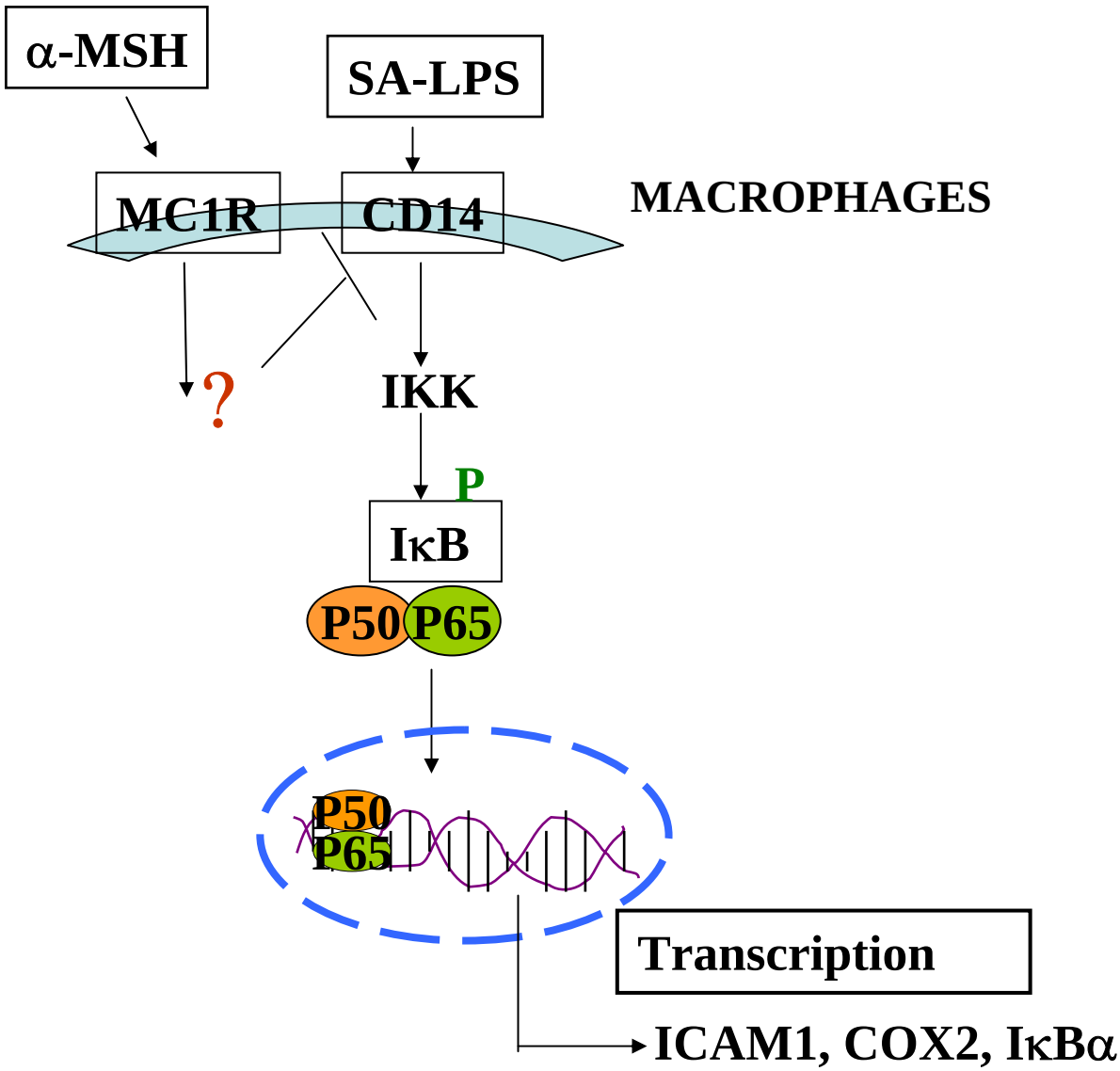
In continuation with our quest to understand the signaling mechanism of α-MSH in various cells of the immune system, we extended our study to neutrophils, one of the most important players in inflammation. Neutrophils are attracted to the site of tissue damage by chemokines, IL-8 being the most important and well studied among them. IL-8 acts through its receptors CXCRI and CXCRII which are abundant on surface of neutrophils. Hence, we investigated the effect of α-MSH on IL-8-induced neutrophils activation. α-MSH was found to inhibit IL-8-induced NF-κB DNA binding and its responsive gene activation in HL-60 derived Neutrophils. It also inhibited IL-8 induced neutrophil migration, oxidative burst response, NO

production, enzyme release such as myeloperoxidase, β-D-glucoronidase and alkaline peroxidase (Fig. 5.2). Our results revealed that α-MSH acts predominantly via its receptor MC-1R and down regulates both the IL-8 receptors -CXCRI and -CXCRII from neutrophil cell surface thereby inhibiting IL-8 mediated biological responses. Neutrophils bear granules that contain mediators of inflammation. A class of such mediators stored in azurophil granules of neutrophils, are serine proteases - cathepsin G, neutrophil elastase and proteinase 3. We observed that inhibitors of serine proteases -PMSF and -CMK, significantly protected α-MSH-mediated inhibition of IL-8-induced biological responses. The serine protease involved was found to be neutrophil elastase. Thus α-MSH somehow led to activation of the Elastase bearing granules leading to its release, which mediated the cleavage of CXCRs thereby inhibiting IL-8 driven neutrophil responses. Activated neutrophils and macrophages liberate proteases to clear the microbial pathogen but a hyper activation in turn damages the surrounding host tissue. The α-MSH with its ability to down regulate the CXCRs from neutrophil cell surface inhibits the migration of neutrophils towards the inflamed site and might help to reduce the suffering of patients in neutrophil driven diseases.

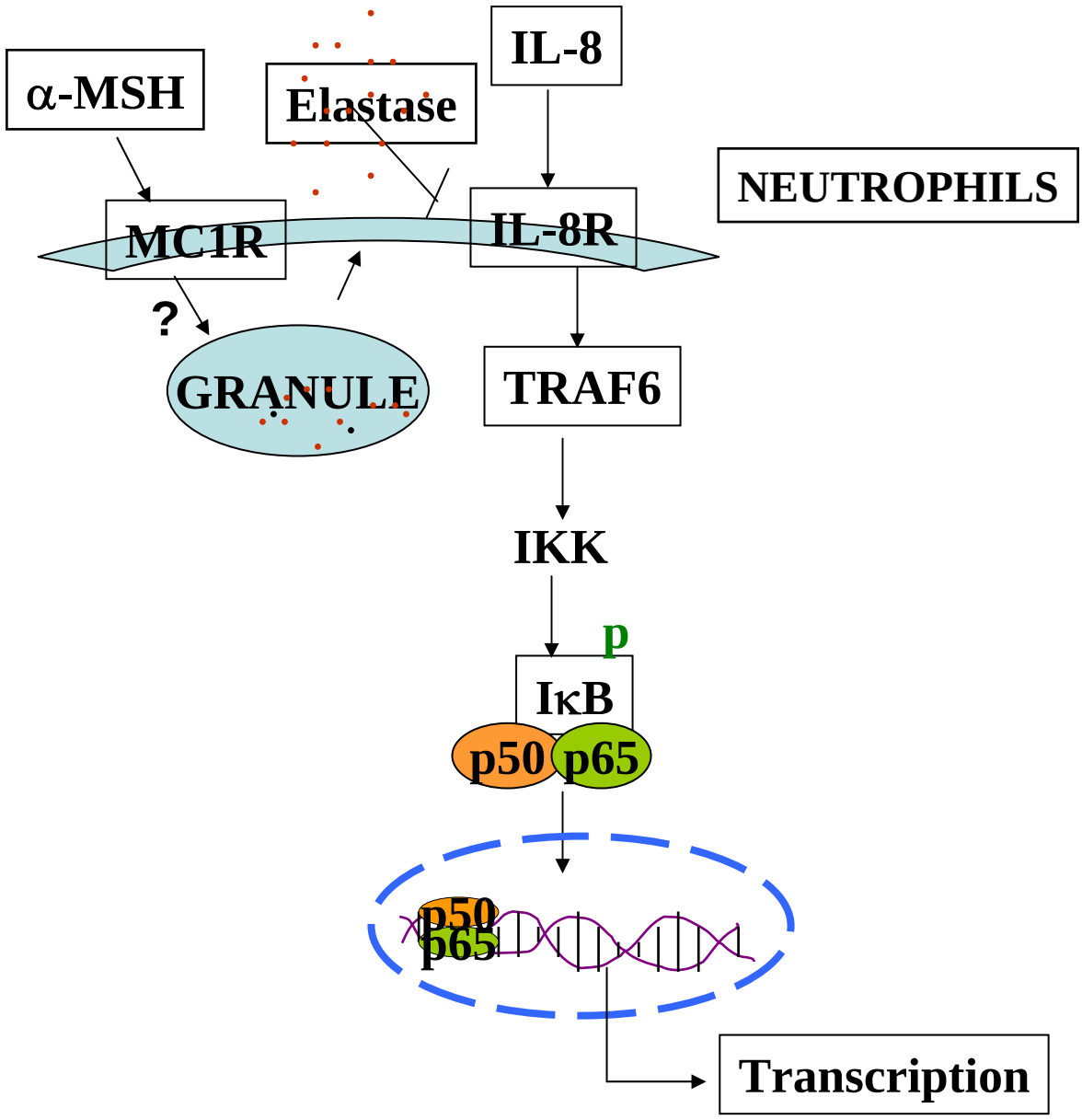
Hypothetical model for action of α-MSH on mast cells



Hypothetical model for action of α-MSH on macrophages



Hypothetical model for action of α-MSH on neutrophils

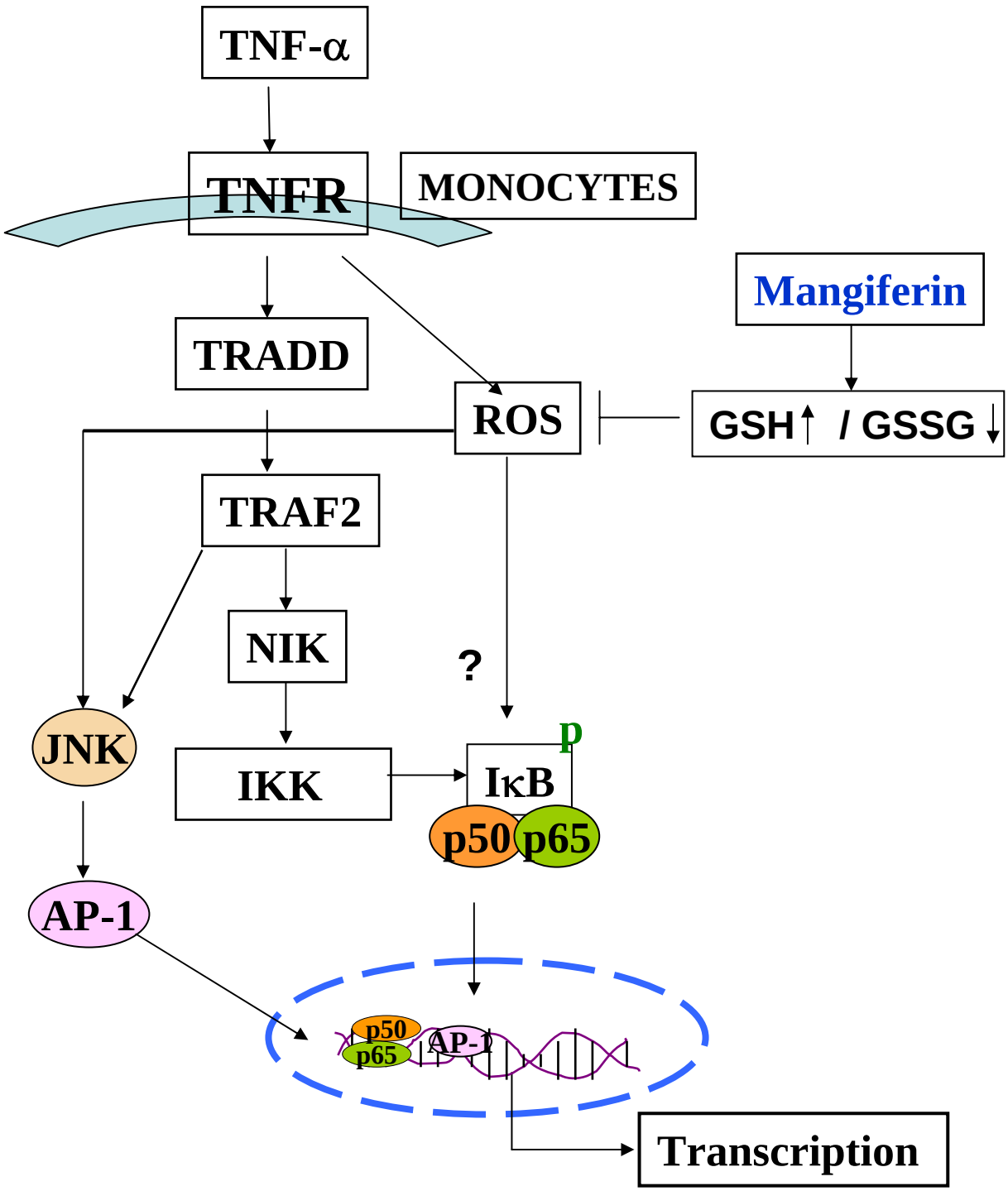


Polyphenols constitute an important group of plant derivatives, which are gaining therapeutic importance in treatment of various inflammatory diseases. One such polyphenol is MANGIFERIN, derived from *Mangifera indica*. Mangiferin has been reported to be used in treatment of several inflammatory diseases in India, China and Cuba. Research so far revealed that mangiferin possesses anti-inflammatory, anti-viral, anti-oxidant, and anti-cancer activities but the mechanism involved is not clear. TNF is a multifaceted cytokine involved in several inflammatory diseases including cancer. We therefore undertook the investigation of effect of mangiferin on TNF-mediated inflammatory responses. Pretreatment of U-937 cells with mangiferin for 3 h led to inhibition of TNF-α induced NF-κB and its responsive genes, ICAM1 and COX2 in a time (beyond 3 h) and dose (10 μg/ml onwards) dependent manner. The effect was mediated through inhibition of IκB kinase (IKK) activation and subsequent blocking of phosphorylation and degradation of IκBα. Overexpression of various signaling molecules in the TNF mediated NF-κB activation pathway in presence or absence of mangiferin, revealed that mangiferin acted at a step upstream of p65 and downstream of IKK (Fig. 6.7.1).

Reactive oxygen species (ROS) are toxic, and in condition of a dysbalance between their overproduction and the diminished activity of various anti-oxidant enzymes and other molecules, induce cellular injury termed as oxidative stress. ROS are often related to a number of inflammatory diseases like atherosclerosis, Alzheimer's, and even cancer. The pathomechanism of ROS mediated damage is not very clear but latest research has shown a connection between ROS and NF-κB. Anti-oxidants, such as N-acetyl cysteine (NAC) and pyrrolidone di-thiocarbamate (PDTC) are reported to quench TNF induced ROI and thereby block TNF mediated NF-κB activation. Mangiferin, similar to these anti-oxidants, led to reduction in the ROI generated by TNF. Important cellular anti-oxidants, well known to be involved in quenching ROI generated are the glutathione and the catalase systems. We found that mangiferin, increases the amount of intracellular glutathione (GSH) and activity of catalase (Fig. 6.8). Thus, mangiferin appears to inhibit the ROI mediated NF-κB activation in response to TNF by increasing the level of cellular GSH, which

quenches the generated ROI. Since Mangiferin inhibited the TNF induced NF-κB, which is an important cell survival factor, we speculated that it might enhance the cell death inducing potential of TNF. As expected we noticed an increase in cell death caused by TNF in presence of mangiferin. TNF-α signaling pathway is an important player in cancerogenesis and hence, a key target for antitumor therapy. The rational is clear. TNF-α induces apoptosis but also upregulates NF-κB, which can drive expression of anti-apoptotic molecules. The inhibition of NF-κB in tumor cells increases the sensitivity of the cells to TNF-α and consequently to chemotherapy. We tested this hypothesis on different standard chemotherapeutic drugs and found mangiferin to be potentiating the cell death induced by all those drugs highlighting mangiferin's potential not only as an anti-oxidant but also in combination therapy.

Hypothetical model for action of mangiferin



Overall the present work helps to understand the signaling mechanism (s) of α-MSH and mangiferin with respect to their anti-inflammatory and anti-tumor activities. The final goal of all pharmaceutical companies is to develop drug(s), which can cure the disease without any side effects. Thus compounds based on endogenous resoluters or naturally occurring plant products seem to be promising in such a search. However, it is very essential to know the molecular mechanism(s) of action of any drug to be successfully employed in order to avoid any unwanted side effects. Hence, in this scenario, the present work brings into light the molecular mechanism(s) of two non-toxic compounds, one an endogenous resoluter and the other, a plant derived product.

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List of Publications and Presentations

Publications:

Yashin Sreenivasan, **Abira Sarkar** and Sunil K. Manna. Oleandrin potentially induces apoptosis CDD

Sunil K Manna*, Yashin Sreenivasan* and **Abira Sarkar**. Cardiac glycoside inhibits IL-8 induced biological responses by downregulating IL-8 receptors through altering membrane fluidity (JCP).

Sunil K Manna*, Yashin Sreenivasan* and **Abira Sarkar**. Cardiac glycoside-induced downregulation of p65 (RelA) is mediated by proteasome, but not by caspases (communicated).

Manna, S. K*., **Sarkar, A*** and Sreenivasan, Y., and (2006). α-Melanocyte Stimulating Hormone downregulates CXC receptors through activation of neutrophil elastase. ***Eur. J. Immu.*** Feb 14; 36(3):754-769.

Abira Sarkar, Yashin Sreenivasan, Govindarajan T. Ramesh, and Sunil K. Manna.(2004) Beta-D-glucoside suppresses TNF-induced activation of nuclear transcription factor kappaB but potentiates apoptosis. **J. Biol. Chem**., 279(32):33768-81.

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Sreenivasan, Y., **Sarkar, A**., and Manna, S. K. (2003): Mechanism of Cytosine arabinoside mediated apoptosis: Role of Rel A (p65) dephosphorylation. ***Oncogene*** 22(28), 4356-4369.

Meetings and Symposiums:

Abira Sarkar, Yashin Sreenivasan Nair, and Sunil K Manna. alpha-Melanocyte Stimulating Hormone induces cell death in mast cells: Involvement of NF-kB. ***XXVIII All India Cell Biology Conference***. ***Pune University.*** January 6-10, 2004.

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