The Mechanisms of Dioscorealide B and Dioscoreanone from the Rhizome of *Dioscorea membranacea* (Pierre ex Prain & Burkill) on Anti-inflammatory Activity in RAW 264.7 Macrophages

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ชื่อวิทยานิพนธ์	กลไกของ Dioscorealide B และ Dioscoreanone จากลำต้นใต้ดิน *Dioscorea membranacea* (Pierre ex Prain & Burkill) ต่อฤทธิ์ต้านการอักเสบใน RAW 264.7 Macrophages
ผู้เขียน	นายพูลสิทธิ์ หิรัญสาย
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บทคัดย่อ

สาร Dioscorealide B และ Dioscoreanone แยกได้จากสารสกัดหยาบส่วนแอลกอฮอล์จากลำต้นใต้ดินของหัวข้าวเย็นชนิด *Dioscorea membranacea* (Pierre ex Prain & Burkill) ที่นิยมใช้เพื่อการรักษาอาการอักเสบและโรคมะเร็งในตำหรับยาแผนไทย มีรายงานว่า Dioscorealide B และ Dioscoreanone มีผลยับยั้งการสร้าง Nitric oxide และ TNF-α จาก RAW 264.7 macrophage ที่ถูกกระตุ้นด้วย LPS ดังนั้นในการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อหากลไกของ Dioscorealide B และ Dioscoreanone ที่มีผลในการยับยั้งการสร้าง Nitric oxide และการแสดงออกของ Cytokine ผ่านทางการส่งสัญญาณของโปรตีนชนิด NF-κB และ ERK1/2 จากการตรวจวัดด้วย Griess's reagent พบว่า Dioscorealide B และ Dioscoreanone สามารถยับยั้งการสร้าง Nitric oxide โดยมีค่า IC_{50} เท่ากับ 2.85 $\pm$ 0.62 μM และ 2.50 $\pm$ 0.64 μM ตามลำดับ และไม่เป็นพิษต่อเซลล์เมื่อตรวจสอบด้วย MTT assay การยังยั้ง Nitric oxide ดังกล่าวเป็นผลมาจาก Dioscorealide B และ Dioscoreanone ไปลดการแสดงออกของ iNOS mRNA และยังสามารถลดการแสดงออกของ IL-1β และIL-6 อย่างมีนัยสำคัญทางสถิติ ที่ความเข้มข้น 6 และ 5 μM ตามลำดับ นอกจากนี้ยังพบว่ามีเพียง Dioscorealide B เท่านั้นที่ยังยั้งการแสดงออกของ IL-10 mRNA ได้ เมื่อศึกษาถึงกลไกการส่งสัญญาณในระดับเซลล์โดยอาศัย pNFκB-Luc reporter system พบว่า Dioscorealide B และ Dioscoreanone ยับยั้งความสามารถในการจับตำแหน่งจำเพาะของโปรตีน NF-κB ซึ่งเป็นผลมาจากการยับยั้งการย่อยสลายตัวของโปรตีนชนิด IκBα ในขณะเดียวกัน Dioscorealide B ในช่วงความเข้มข้น 1.5-6.0 μM ยังสามารถยับยั้งการทำงานของโปรตีนชนิด ERK1/2 ส่วน Dioscoreanone ในช่วงความเข้มข้น 1.2-5.0 μM มีผลส่งเสริมการกระตุ้นการทำงานของโปรตีนชนิด ERK1/2 นอกจานี้ Dioscoreanone เพียงอย่างเดียวยังสามารถกระตุ้นการทำงานของ ERK1/2 ตามความเข้มข้นและระยะเวลาที่เพิ่มขึ้น โดยเป็นผลมาจากการเกิดกระบวนการ Arylating reaction และกระบวนการ Redox cycling reaction ของเอนไซม์ NQOR ซึ่งทั้งสองกระบวนการสามารถยับยั้งได้ด้วยสาร *N*-acetyl cysteine และสาร Dicoumarol ตามลำดับ จากการศึกษาครั้งนี้สรุปได้ว่ากลไกของ Dioscorealide B ในการยับยั้งการสร้าง Nitric oxide และการแสดงออกของ iNOS, IL-1β, IL-

6 และ IL-10 เป็นผลมาจากการยับยั้งสัญญาณไปยังโปรตีนชนิด NF-κB และ ERK1/2 ส่วน Dioscoreanone ยับยั้งการสร้าง Nitric oxide และการแสดงออกของ iNOS, IL-1β และ IL-6 เป็นผลมาจากการต้านการส่งสัญญาณไปยังโปรตีนชนิด NF-κB และการคงระดับการแสดงออกของ IL-10 mRNA โดย Dioscoreanone เป็นผลมาจากการกระตุ้นการทำงานของโปรตีนชนิด ERK1/2 ข้อมูลที่ได้จากการศึกษาครั้งนี้แสดงให้เห็นถึงฤทธิ์ต้านการอักเสบของ Dioscorealide B และ Dioscoreanone ซึ่งช่วยสนับสนุนองค์ความรู้แพทย์แผนไทยในการใช้หัวข้าวเย็นชนิด *Dioscorea membranacea* ในการรักษาอาการอักเสบ

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ABSTRACT

Dioscorealide B and Dioscoreanone have been purified from an ethanol extract of the rhizome of *Dioscorea membranacea* (Pierre ex Prain & Burkill) which has been used to treat inflammation and cancer in Thai Traditional Medicine. Previously, Dioscorealide B and Dioscoreanone have been reported to have anti-inflammatory activities through reducing nitric oxide (NO) and Tumor Necrosis Factor-α (TNF-α) production in LPS-induced RAW 264.7 macrophage cells. In this study, the mechanisms of Dioscorealide B and Dioscoreanone on LPS-induced NO production and cytokine expression through the activation of NF-κB and ERK1/2 were demonstrated in RAW 264.7 cells. Through measurement with Griess's reagent, Dioscorealide B and Dioscoreanone reduced NO level with an IC_{50} value of 2.85±0.62 μM and 2.50±0.64 μM, respectively. The inhibition of NO production by Dioscorealide B and Dioscoreanone was due to the significant suppression of LPS-induced iNOS mRNA expression as well as IL-1β and IL-6 mRNA at a concentration of 6.0 and 5.0 μM, respectively. Only Dioscorealide B was found to inhibit IL-10 mRNA expression. At the signal transduction level, Dioscorealide B and Dioscoreanone significantly inhibited NF-κB binding activity, as determined using pNFκB-Luciferase reporter system, which resulted from the prevention of IκBα degradation. In addition, Dioscorealide B in the range of 1.5-6.0 μM significantly suppressed the activation of the ERK1/2 protein, whereas Dioscoreanone in the range of 1.2-5.0 μM significantly enhanced LPS-induced ERK1/2 activation. Dioscoreanone alone could induce the activation of ERK1/2 proteins in a concentration- and time-dependent response. The activation of ERK1/2 proteins by Dioscoreanone was mediated by arylating reaction and redox cycling reaction of NQOR that were inhibited by *N*-acetyl cysteine and Dicoumarol, respectively. In conclusion, the mechanisms of Dioscorealide B on the inhibition of NO production and mRNA expression of iNOS, IL-1β, IL-6 and IL-10 were

due to the inhibition of NF-κB and MAPK/ERK signaling pathway in LPS-induced RAW264.7 macrophage cells. In contrast, Dioscoreanone inhibited NO production and mRNA expression of iNOS, IL-1β and IL-6 that were due to the inhibition of NF-κB activation, and the constant expression of IL-10 in the presence of Dioscoreanone may result from the ERK1/2 stimulation. This study demonstrates the anti-inflammatory activity of Dioscorealide B and Dioscoreanone that supports the use of *Dioscorea membranacea* rhizome in Thai Traditional Medicine.

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LIST OF ABBREVIATION AND SYMBOLS

×g	=	Relative gravity force
°C	=	Degree Celsius
ΔC	=	C-terminal deletion
μg	=	Micrograms
μl	=	Microliters
μm	=	Micrometers
μM	=	Micromolar or micromole/liter
AA	=	Arachidonic acid
Ab	=	Antibody
ANOVA	=	Analysis of variance
APS	=	Ammoniumpersulfate
ATCC	=	American type culture collection
ATP	=	Adenosine triphosphate
BH_4	=	Tetrahydrobiobterin
bp	=	Base pairs
BSA	=	Bovine serum albumin
CaM	=	Calmodulin
CD	=	Cluster of differentiation
cDNA	=	Complementary DNA
cm^2	=	Squear centimeters
COX	=	Cyclooxygenases
CpG	=	Cytosine-phosphate-Guanine
CR	=	Complement receptor
CREB	=	cAMP response element-binding protein
CSF	=	Colony-stimulating factor
dATP	=	Deoxyadenosine-5'-triphosphate
DC	=	Dendritic cell
dCTP	=	Deoxycytidine-5'-triphosphate
DEPC	=	Diethyl pyrocarbonate
dGTP	=	Deoxyguanosine-5'-triphosphate
DI	=	Deionized water

LIST OF ABBREVIATION AND SYMBOLS (CONTINUED)

Dic	=	Dicoumarol
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleoside-5'-triphosphate
DTH	=	Delayed type hypersensitivity
DTT	=	Dithiothreital
dTTP	=	Deoxythymidine-5'-triphosphate
DW	=	Distilled water
ECIST	=	Evolutionarily conserved signaling intermediate in Toll pathway
ECM	=	Extracellular matrix
EDTA	=	Ethylenedaminetetraacetic acid
ERK	=	Extracellular signal-regulated kinase
ESR	=	Erythrocyte sedimentation rate
et al.	=	et ali (Latin) and others
FAD	=	Flavin adenine dinucleotide
FBS	=	Fetal bovine serum
Fc	=	Fragment crystallization region
FcR	=	Fc receptor
FGF	=	Fibroblast growth factor
FMN	=	Flavin mononucleotide
FW	=	Formular weight
F-primer	=	Forward-primer
GRR	=	Glycine rich region
GSH	=	Glutathione
HETE	=	5-hydroxyeicosatetraenoic acid
HIV	=	Human immunodeficiency virus
HLH	=	Helix-loop-helix motif
HMWK	=	High-molecular weigh kininogen
HPETE	=	5-hydroperoxyeicosatetraenoic acid
HRP	=	Horseradish peroxidase

LIST OF ABBREVIATION AND SYMBOLS (CONTINUED)

hrs	=	hours
HSP	=	Heat shock protein
IC50	=	The 50% inhibitory concentration
ICAM	=	Intercellular adhesion molecule
IFN	=	Interferon
Ig	=	Immunoglobulin
IκB	=	Inhibitor of κB
IKK	=	Inhibitor of κB kinase
IL	=	Interleukin
IL-1R	=	Interleukin-1 receptors
IRAK	=	IL-1R-associated kinase
IRF	=	Interferon regulatory factor
JNK	=	c-Jun *N*-terminal kinase
KD	=	Kinase domain
kDa	=	Kilo Daltons
LFA	=	Leukocyte function-associated antigen
LPS	=	Lipopolysaccharide
LRR	=	Leucine-rich repeat
LTB	=	Leukotriene B
LTC	=	Leukotriene C
LTD	=	Leukotriene D
LTE	=	Leukotriene E
LZ	=	Leucine-zipper motif
Mac	=	Macrophage antigen
MAC	=	Membrane attack complex
MAPK	=	Mitogen-activated protein kinase
MD-2	=	Lymphocyte antigen 96
MEK	=	MAPK/ERK kinase
MEKK	=	MAPK/ERK kinase kinase
mg	=	Milligrams
min	=	Minutes

LIST OF ABBREVIATION AND SYMBOLS (CONTINUED)

MIP	=	Macrophages inflammatory protein
MKK	=	MAPK kinase
ml	=	Milliliter
M-MLV	=	Moloney murine leukemia virus
MMMTV	=	Mouse mammary tumor virus
mRNA	=	Messenger RNA
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MyD88	=	Myeloid differentiation factor 88
NAC	=	*N*-acetyl cysteine
NADPH	=	Nicotinamide adenine dinucleotide phosphate reduced form
NED	=	*N*-1-napthylethelenediamine dihydrochloride
NEMO	=	NF-κB essential modulator
NES	=	Nuclear export signal
NF-κB	=	Nuclear factor-κB
NK	=	Natural killer
NLS	=	Nuclear localizing sequence
nm	=	Nanometers
NMR	=	Nuclear magnetic resonance
NOS	=	Nitric oxide synthase
NQOR	=	NAD(P)H: Quinone oxidoreductase
NSAID	=	Nonsteroidal anti-inflammatory drugs
OD	=	Optical density
p	=	p value
PAF	=	Platelet-activating factor
PAR	=	Protease-activated receptor
PARP	=	Poly (ADP-ribose) polymerase
PBS	=	Phosphate buffer saline
PDGF	=	Platelet-derived growth factor
PECAM	=	Platelet endothelial cell adhesion molecule
PGD	=	Prostaglandin D

LIST OF ABBREVIATION AND SYMBOLS (CONTINUED)

PGE	=	Prostaglandin E
PGI	=	Prostaglandin I
PI3K	=	Phosphoinositide-3 kinase
PKC	=	Protein kinase C
PKR	=	Protein kinase R
pNFκB-Luc	=	Plasmid containing luciferase gene under the control of NF-κB binding motif
Poly (I:C)	=	Polyinosinic: Polycytidylic acid
PRR	=	Pattern-recognition receptor
PTPase	=	Protein tyrosine phosphatase
RANTES	=	Regulated upon activation, normal T-cell expressed and secreted
RHD	=	Rel-homology domain
RIP	=	Receptor-interacting protein
RIPA	=	Radio-immunoprecipitation assay
RNA	=	Ribonucleic acid
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
rpm	=	Revolution per minute
RSV	=	Respiratory syncytial virus
RT-PCR	=	Reverse transcriptase-polymerase chain reaction
R-primer	=	Reverse-primer
sec	=	Seconds
S.E.M.	=	Standard error mean
SCF	=	SKp1-Culin-F-box
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	SDS-polyacyramide gel electrophoresis
SOD	=	Superoxide dismutase
TAK	=	TGF-β-activated kinase
Taq	=	*Thermos aquaticus*
TBE	=	Tris-borate, EDTA

LIST OF ABBREVIATION AND SYMBOLS (CONTINUED)

TBK	=	TRAF-family member-associated NF-κB activator-binding kinase
TBP	=	TATA-binding protein
TBS	=	Tris-buffered saline
TD	=	Tranactivation domain
TEMED	=	N,N,N',N'-tetramethylethylenediamine
TGF	=	Transforming growth factor
TIR	=	Toll/IL-1 receptor
TIRAP	=	TIR-associated protein
TLR	=	Toll-like receptor
Tm	=	Melting temperature
TNF	=	Tumor necrosis factor
TRAF	=	TNF receptor-associated factor
TRAM	=	TRIF-related adaptor molecule
TRCP	=	β-transduction repeat-containing protein
TRIF	=	TIR domain-containing adaptor inducing interferon-b
TTBS	=	Tween 20-TBS
V	=	Volt
v/v	=	Volume per volume
VCAM	=	Vascular cell adhesion molecule
VEGF	=	Vascular endothelial growth factor
VLA	=	Very late antigen
w/v	=	Weight per volume
WBC	=	White blood cells

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Inflammation is the physiological response within the human body to tissue injury induced by inflammatory stimuli (Sommer, 2002). The inflammatory responses are initiated and amplified by the production of mediators from immune cells such as arachidonic metabolites, nitric oxide, pro-inflammatory cytokines and chemokines. Although inflammation serves a protective function of host, uncontrolled inflammation results in vasodilation, increment of vascular permeability, fever, massive tissue destruction, multi organ dysfunction and shock (Rosenberg and Gallin, 2003). In inflammatory therapy, anti-inflammatory drugs that are used to manage the inflammatory condition include steroidal and non-steroidal agents. The non-steroidal anti-inflammatory drugs (NSAID) are used extensively to manage pain and inflammation associated with surgical procedures, inflammatory diseases such as osteoarthritis and rheumatoid arthritis, and more modulate pain associated with migraines, dysmenorrheal, myalgia and dental pain. The steroidal anti-inflammatory agents exert more efficiency in anti-inflammation than NSAID and show different mechanisms. NSAID inhibit cyclooxygenase activity, whereas steroids reduce arachidonic acid level, the substrate of cyclooxygenases. However, both steroidal and non-steroidal agents exert a broad specificity of anti-inflammatory responses and can induce side effects to host such as prothrombotic induction leading to a greater incidence of acute coronary artery disease, gastroenteropathy, osteoporosis and increased risk of infection (Hansen, 1998).

In Thailand, plants named Hua-Khao-Yen were included in the multi-medicinal plants formula of more than 2,449 recipes based on the selective interview from 23 folk medicinal doctors (Itharat, 2002). These recipes were used traditionally in the treatment of bone and joint diseases (26.1%), cancer related inflammation (43.5%) and other diseases such as lymphopathy, dermopathy, venereal diseases and leprosy. The methods of preparation were decoction (69.6%) and maceration (30.4%). Using scientific methods, Hua-Khao-Yen was identified into five species: *Smilax corbularia*, *Smilax glabra*, *Dioscorea birmanica*, *Dioscorea membranacea* and *Pygmeopremna herbacea*.

Previously, the aqueous and ethanol extracts of all Hua-Khao-Yen had been determined as anti-inflammation via the inhibition of LPS-induced Nitric Oxide and tumor necrosis factor-α (TNF-α) productions. The results showed that *Dioscorea membranacea* extracts exhibited the more potency in this inhibitory activity and the effective compounds of this action were Dioscorealide B and Dioscoreanone (Tewtrakul and Itharat, 2007).

The goal of anti-inflammatory drug development continues to be essential not only in terms of the need for compounds that prevent inflammatory symptoms but also the identification of their mechanisms. Dioscorealide B and Dioscoreanone have shown their effect on nitric oxide and TNF-α productions but their mechanisms are not identified. Therefore, the mechanisms of Dioscorealide B and Dioscoreanone on the activation of the NF-κB and ERK1/2 MAPK signaling pathways correlate to induce Nitric oxide production and expression of inflammatory mediators including iNOS, IL-1β, IL-6 and IL-10 in LPS-induced RAW 264.7 murine macrophages.

1.2 Review of Literature

1.2.1 Immune Responses

The immune response is a collective and coordinated response of cells and molecules in the immune system to the introduction of foreign substances. Defense against foreign bodies is mediated by the early reaction of innate immunity and the later responses of adaptive immunity (Abbas and Lichtman, 2003). The feature of these responses is characterized by the specificity, diversity, memory and components (Table 1).

The innate or natural or native immunity consists of cellular and biochemical defense mechanisms that are normally in place even before infection and poised to respond rapidly to infection. These mechanisms react only to microbes and not to noninfectious substances, and they respond in the same way to repeated infection. The principal components of innate immunity are (1) physical and chemical barriers; (2) phagocytic cells (neutrophils and macrophages) and natural killer (NK) cells; (3) plasma proteins, including complement system and inflammatory mediators; and (4) cytokines that regulate and coordinate innate immune cell activity. The mechanisms of innate immunity are specific for structures that are common to the group of related microbes and may not distinguish fine differences between foreign substances (also called pattern recognition).

Innate immunity is the first line of defense and the inflammatory response is the component of innate immunity (Abbas and Lichtman, 2003).

In contrast to innate immunity, adaptive immunity is stimulated by exposure to infectious agents and increases in magnitude and defensive capabilities with each successive exposure to particular microbes. The defining characteristics of adaptive immunity are exquisite specificity for distinct molecules and ability to remember and respond more vigorously to repeated exposures to the same microbes. The main components of adaptive immunity are lymphocytes and their products. These components have an extraordinary capacity to distinguish among different, closely related microbes and molecules and for this reason it is also called specific immunity. In addition, this response may also be called acquired immunity which requires experience to initiate the potent protective actions (Abbas and Lichtman, 2003).

Innate and adaptive immune responses are components of an integrated system of host defense in which numerous cells and molecules function cooperatively. The innate immune response to microbes stimulates adaptive immune responses and influences the nature of the adaptive immune responses. Conversely, the adaptive immune responses use many effecter mechanisms of the innate immune response to eliminate foreign bodies, and they function by enhancing the destroying activities of innate immune mechanisms (Abbas and Lichtman, 2003).

Table 1. Features of innate and adaptive immunity (Abbas and Lichtman, 2003)

	Innate Immunity	Adaptive Immunity
Characteristics		
Specificity	- For structures shared by groups of related microbes	- For antigens of microbes or nonmicrobial antigens
Diversity	- Limited - Germline-encoded	- Very diverse - Receptors are produced by somatic recombination of gene segments
Memory	No	Yes
Nonreactive to self	Yes	Yes
Components		
Physical and chemical barriers	- Skin, mucosal epithelia - Antimicrobial chemical	- Lymphocytes in epithelia - Antibodies secreted at epithelial surfaces
Blood proteins	- Complement	- Antibodies
Cells	- Phagocytes (macrophages, neutrophils) - natural killer cells	- Lymphocytes

1.2.2 Inflammation

Cells can be injured by many different agents and mechanisms, and cellular injury is the common denominator in all diseases. Inflammation is an innate immunity (Peterson et al., 1995; Kumar et al., 2007) which works together with the physiological process of vascularized tissue (Sommer, 2002; Rosenberg and Gallin, 2003), intended to eliminate the initial cause of cell injury as well as the necrotic cell and tissues. The inflammatory response has three purposes: (1) to neutralize and destroy invading and harmful agents, (2) to limit the spread of harmful agents to other tissue, and (3) to prepare any damaged tissue for repair. Although inflammation is a nonspecific immune response, this reaction is involved in the adaptive immune response. Inflammatory reactions are required to differentiate self from non-self antigens and transfer signals to initiate adaptive immune responses. When adaptive immunity is induced, it releases chemical mediators to enhance inflammatory cells in the elimination of non-self antigens. When dysfunctional, the immune responses may contribute to the mechanism of disease. Inflammatory processes may contribute to distressing clinical manifestations of disease at the same time they are helping to eradicate it. In some cases, such as when inflammation is excessive or when it occurs in enclosed spaces (joints or cranium), inflammation may cause additional cellular injury. In systemic inflammatory response syndrome, a hypermetbolic response often results in multiple organ failure. The suffix "*-itis*" is commonly used to describe conditions that exhibit inflammation. For example, appendicitis, tendinitis and nephritis refer to inflammation of the appendix, tendon and kidney, respectively (Peterson et al., 1995).

1.2.2.1 Clinical Signs of Inflammation

A. Cardinal Clinical Signs

Five cardinal clinical signs of inflammation are redness (rubor), swelling (tumor), heat (calor), pain (dolor) and loss of function (funtiolaesa) as described since the first century of the Common Era. Redness and heat are due to increased blood flow to the inflamed area. Swelling is due to an accumulation of fluid. Pain is due to a release of chemicals that stimulate nerve ending. And, loss of function is due to a combination of factors. These signs are commonly manifested when inflammation occurs on the surface of

the body, but not all of them will be apparent in inflammation of internal organs. Pain occurs only when there are appropriate sensory nerve endings in the inflamed site. For example, inflammation of the lung does not cause pain unless the inflammation involves the parietal pleura, where there are pain-sensitive nerve endings. The increased heat of inflamed skin is due to the entry of a large amount of blood at body core temperature into the normally cooler skin. When inflammation occurs internally where tissue is normally at body temperature, no increase in heat is apparent (Rosenberg and Gallin, 2003).

B. Systemic Clinical Signs

The inflammation may be accompanied by systemic features in addition to the local cardinal signs. The systemic clinical signs contain fever, changes in the peripheral white blood cell count, and changes in plasma protein levels. Fever may result from the entry of pyrogens and inflammatory mediators into the circulation at the site of inflammation. These act upon the brain stem to reset body temperature. In peripheral blood circulation, the total number of neutrophils in the peripheral blood increases (neutrophil leukocytosis). Initially, this reaction is due to an accelerated release of neutrophils from bone marrow. Later, neutrophil production in bone marrow is increased. Peripheral blood neutrophils tend to be less mature forms and they frequently contain large cytoplasmic granules (toxic granulation). Viral infections tend to produce neutropenia (decreased number of neutrophils in the blood) and lymphocytosis (excess of normal lymphocytes in the blood). In addition, the levels of certain plasma proteins typically increase when inflammation is present. These acute phase reactants include C-reactive protein, $\alpha 1$-antitrypsin, fibrinogen, haptoglobin and ceruloplasmin. Increased levels of these substances in turn lead to an increased erythrocyte sedimentation rate (ESR), a simple and useful (though nonspecific) clue to the presence of inflammation (Chandrasoma and Taylor, 1998).

1.2.2.2 Causes of Inflammation

As described above, inflammation is commonly evoked in response to tissue injury. Injury can arise from sources outside the body or from sources inside the body. The endogenous injurious agents result from tissue ischemia, immune reaction to infectious

agents, endogenous foreign bodies. Surgery, trauma, burns and skin injury from chemicals are examples of the exogenous agents. Tissue necrosis includes ischemia from myocardial infraction or pulmonary embolism, physical injury from burns or freezing, and chemical injuries from acidic or basic agents. Infections from bacteria, virus, fungi or parasites are among the most common and medically important causes of inflammation. An immune attack against a specific antigen results in the activation of immune cells and secretion of cytokines which mediate an inflammatory response even in the absence of specific cellular damages. In addition, the environmental substances and self tissue can induce immune reaction (called hypersensitivity reaction) that progress to inflammation. Because these stimuli mostly can not be eliminated, such reactions tend to be persistent and are important causes of morbidity and mortality. Each of these stimuli may induce reactions with some distinctive characteristics but all inflammatory reactions have the same basic features (Kumar et al., 2007).

1.2.2.3 Types of Inflammation

Inflammation is traditionally categorized as either acute or chronic. Acute inflammation occurs rapidly (within minutes), short in duration (lasting less than 2 weeks) and involves a discrete set of events. The acute inflammatory responses are relatively uniform and characterized by accumulations of fluid, plasma proteins and granulocytic leukocytes. In contrast, chronic inflammation is of longer duration from a day to years. The characteristic of chronic inflammation is typified by an influx of lymphocytes and macrophages with associated vascular proliferation and fibroblast growth. Chronic inflammation results in the formation of scar tissue and deformity or progression to inflammatory-related disorders. However, these basic formations of both acute and chronic inflammations can overlap, and many variables modify their cause and histological appearance (Peterson et al., 1995; Rosenberg and Gallin, 2003).

1.2.2.4 Inflammatory Responses

Although the inflammatory response can be initiated by a variety of injurious agents and the extent can vary, the sequence of events that follows is remarkably similar. As shown in Fig. 1, the inflammatory responses are initiated by tissue injury that is

induced by non-immune or immune stimuli. The first visible tissue change begins immediately after an injury. It is the microcirculatory response accompanied by mobilization of acute inflammatory response. The immune response is triggered at the time of the injury but takes several days to manifest microscopically visible changes at the site of injury. Events in the acute inflammatory reaction process include vascular responses and cellular responses. These reactions are accompanied with the inflammatory mediators that are produced during tissue injury and initiate the acute phase response. The term of chronic inflammatory response is applied to the complex changes in tissues that represents a combined inflammatory and immune response against an antigen that persists in the tissues long enough so that microscopic changes of the immune response can appear. Chronic inflammation shows changes associated with tissue damage and repair (Rosenberg and Gallin, 2003).

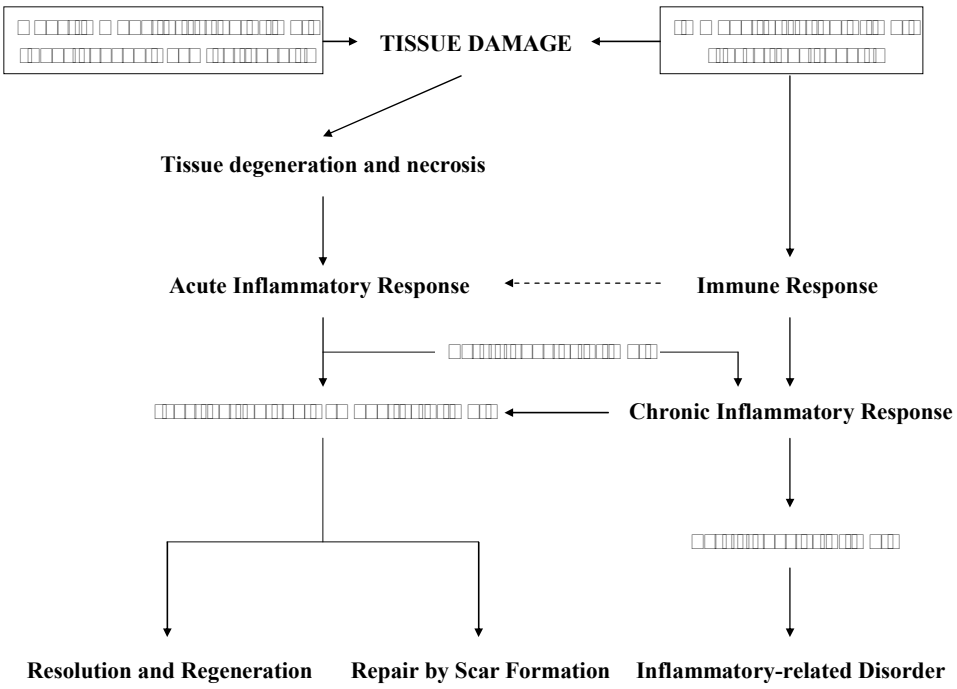

Figure 1. Summary of host response to tissue injury

A. Acute Inflammatory Responses

An acute inflammatory response is characterized by a rapid onset and resolution of tissue change and damage in a short period. Changes occur locally at the site of the injury as well as systemically. A general alarm and recruitment system sent throughout the body is known as the acute phase response. A rapid increase and decrease in several plasma proteins is characteristic of the acute phase proteins that function to increase inflammation through activation of complement. The manifestation of acute inflammation can be divided into categories: vascular and cellular responses. At the biochemical level, many of the responses that occur during acute inflammation are associated with the release of chemical mediators. The hemodynamic and white blood cell responses contribute to the inflammatory exudates, the extravascular influx of fluid containing high concentrations of proteins, salts, cell and cellular debris (Peterson et al., 1995; Sommer, 2002; Kumar et al., 2007).

A.1 Vascular Responses

The vascular or hemodynamic changes that occur with inflammation begin almost immediately after injury and are initiated by a momentary constriction of small vessels in the area. After transient vasoconstriction (lasting only for seconds), vasodilation of arterioles and venules occurs, resulting in locally increased blood flow and engorgement of down-stream capillary bed. This vascular expansion is the cause of the redness (erythema) and warmth associated with the cardinal clinical signs of inflammation. As the microvasculature becomes more permeable, protein-rich fluid moves into the extravascular tissues. This causes the red blood cells to become more concentrated, thereby increasing blood viscosity and slowing circulation. These changes are reflected microscopically by numerous dilated small vessels packed with erythrocytes and slow flowing blood, a process called *stasis*. As stasis develops, this process enhances the cellular response (Sommer, 2002).

In the early phase of acute inflammation, arteriolar vasodilation and increased volume of blood flow lead to a rise in intravascular hydrostatic pressure, resulting in movement of fluid from capillaries into the tissue. This fluid, called *transudate*, is essentially an ultrafiltrate of blood plasma and contains little protein. However, transudation

is soon eclipsed by increasing vascular permeability that allows the movement of protein-rich fluid and even cells (called exudate) into the interstitium. The loss of protein-rich fluid into the perivascular space reduces the intravascular osmotic pressure and increases the osmotic pressure of interstitial fluid. The net result is an outflow of water and ions into the extravascular tissues. Fluid accumulation in extravascular spaces is called edema (swelling). The fluid may be transudate or exudate (Kumar, 2007).

These responses benefit the host by controlling the effects of the injurious agent. The movement of fluid out of the capillaries and into the tissue spaces dilutes the toxic and irritating agent. As fluid moves out of the capillaries, stagnation of flow and clotting of blood in the small capillaries occurs at the site of the injury. This aids in localizing the spread of infectious microorganisms (Peterson et al., 1995).

Depending on the severity of the injury, the hemodynamic changes that occur with inflammation follow one of three patterns of responses. The first is an immediate transient response, which occurs with minor injuries. The second is an immediate sustained response, which occurs with more serious injuries and continues for several days and damages the vessels in the area. The third type of response is a delayed hemodynamic response; the increase in capillary permeability occurs 4 to 24 hours after injury. A delayed response often accompanies radiation types of injuries, such as sunburn (Peterson et al., 1995; Sommer, 2002; Kumar et al., 2007).

A.2 Cellular Responses

An important function of the inflammatory response is to deliver leukocytes to the site of the injury and to activate them. Leukocytes ingest offending agents, kill bacteria and other microbes, and eliminate necrotic tissue and foreign substances. Leukocytes that participate in acute inflammatory response mostly are granulocytes and mononuclear phagocytes.

A.2.1 Component of Cellular Response

a. Granulocytes

Granulocytes are identifiable because of their characteristic cytoplasmic granules. These white blood cells have distinctive multi-lobed nuclei. The granulocytes are divided into three types according to the staining properties of the granules such as neutrophils, eosinophils and basophils.

Neutrophil is the primary phagocyte that arrives early at the site of inflammation, usually within 90 minutes of injury. Their cytoplasmic granules contain enzymes and other antibacterial substances that are used in destroying and degrading the engulfed particles. They also have oxygen dependent metabolic pathways that generate toxic oxygen and nitrogen products. Because these white blood cells have nuclei that are derived into three or five lobes, they are often called *polymorphonuclear neutrophils* or *segmented neutrophils*. The neutrophil count in the blood often increases greatly during the inflammatory process, especially with bacteria infections. After being released from bone marrow, circulating neutrophils have a life span of approximately 10 hours and, therefore, must be constantly replaced if their numbers are to remain adequate. This requires an increase in circulating white blood cells, a condition called leukocytosis. There are several products that participate in the process of neutrophil maturation and activation (Table 2). With excessive demand for phagocytes, immature forms of neutrophils are released from bone marrow. These immature cells are often called bands because of the horseshoe shape of their nuclei. The phase *a shift to the left* in a white blood cell differential count refers to the increase in immature neutrophils seen in severe infections.

Eosinophils are primarily tissue-dwelling and are recruited to sites of acute inflammation, seen most prominently in response to respiratory, gastrointestinal and dermatological allergens and in response to generalized infections with helminthic parasites. Like neutrophils, eosinophils develop in bone marrow. They have receptor-mediated responses to specific activating agents, including RANTES (the chemokines regulated upon activation normal T-cell expressed presumably secreted), macrophage inflammatory protein (MIP)-1α, eotaxin and IL-5. The cytoplasmic granules of eosinophils contain oxidative and cationic proteins. In contrast to neutrophils, eosinophils are ineffective phagocytes and release the contents of their granules to the extracellular milieu. Interestingly, the

detrimental features of eosinophils, particularly their contributions to the pathogenesis of reactive airway disease, are among the best characterized, whereas the beneficial aspects of eosinophils in the inflammatory response remain poorly understood. A role of eosinophils in host defense against respiratory virus pathogens in turn suggests the possibility that asthma and respiratory allergy represent the detrimental side of what has been designed as innate, eosinophils-mediated antiviral host defense.

Basophil and mast cell are derived from CD34+ hematopoietic progenitor cells in the bone marrow. Basophil mostly presents 0.5 to 1% of blood leukocytes, whereas mast cells are found in the connective tissue beneath epithelia and near blood vessels and nerves. Mast cells can be classified into two major subsets depending on their anatomic location, granule contents and activity, including connective tissue mast cell and mucosal mast cell. The activities of the latter are dependent on T-cells. Although basophils are normally not present in tissue, they are recruited to some inflammatory sites, usually together with eosinophils. The average basophil life span is measured in days, while mast cells can live weeks to months. Basophils and mast cells are capable of synthesizing many of the same mediators such as vasoactive amines, proteolytic enzymes, proteoglycan, lipid mediators and cytokines. Like mast cells, basophils bind IgE and are triggered by antigens binding to the IgE. Therefore, basophils and mast cells may contribute to immediate hypersensitivity reactions. Degranulation of mast cells and basophils begins the inflammatory responses and is characteristically associated with an allergy or Type I hypersensitivity reaction. However, mast cells and basophils are also involved in wound healing and chronic inflammatory conditions.

Table 2. Agents promoting neutrophil activation (Rosenberg and Gallin, 2003)

Agents	Function stimulated
Leukotriene B_4 (LTB_4)	- Chemoattractant - Enhances adherence to endothelial cells - Activates degranulation and NADPH oxidase activity
Complement fragment C5a	- Chemoattractant - Induces degranulation and adherence
Platelet activating factor	- Induces aggregation and adherence - Chemoattractant and degranulation
Histamine	- Concentration-dependent changes in chemotaxis - Neutrophil priming - Degranulation
Interferon-γ	- Increases antibody-dependent cytotoxicity - Neutrophil Priming
Granulocyte-CSF	- Increases antibody-dependent cytotoxicity and - Neutrophil Priming - Enhances phagocytosis - Stimulates maturation within bone marrow
Granulocyte-macrophage CSF	- Neutrophil Priming - Stimulates maturation within bone marrow

Table 2. Agents promoting neutrophil activation (cont.)

Agents	Function stimulated
Tumor necrosis factor-α	- Chemoattractant - Neutrophil Priming - Enhances phagocytosis and antibody-dependent cytotoxicity
Interleukin-8 (IL-8)	- Chemoattractant - Induces degranulation and NADPH activity
f-Met-Leu-Phe	- Induces aggregation, degranulation and NADPH activity
Fibrinogen	- Primes neutrophils - IL-8 synthesis in response to f-Met-Leu-Phe and LTB_4

b. Monocytes and Macrophages

The monocytes are the largest of the white blood cells and constitute 3 to 8% of the total blood leukocytes. The circulating life span of the monocytes is three or four times longer than that of the granulocytes, and these cells survive for a longer time in the tissues. The monocytes, which migrate in increased numbers into the tissues in response to inflammatory stimuli, mature into macrophages. Examples of tissue macrophages include the Kuffer cells of the liver, the alveolar macrophages (type I cells) of the lungs and microglial cells of the brain. Within 5 hours, mononuclear cells arrive at the inflammatory site, and by 48 hours, monocytes and macrophages are the predominant cell types. Macrophages can be activated by lymphokines, immune complexes and complement protein (C3b). Macrophages are powerful phagocytes, each capable of destroying bacteria. They can engulf larger and greater quantities of foreign material than the neutrophils. Macrophages can survive for many months after destroying bacteria. Because of their phagocytic and debridement functions, macrophages are the predominant phagocytic cells in late inflammation. Not only do they ingest particulate matter, macrophages migrate to the local lymph node and present antigens to lymphocytes, initiating the specific immune responses. These activated lymphocytes play an important role in chronic inflammation, where they can surround and wall off foreign material that can not be digested. In addition, macrophages release chemical mediators that are involved in inflammation and stimulate wound healing.

c. Endothelial Cells

Endothelial cells line the blood vessels, serving as a critical interface between the blood and the body tissues. These cells sense physical (hemodynamic) and chemical (vasoactive mediators) stimuli and respond by synthesizing and secreting a variety of small molecules, lipids and proteins (Table 3). These endothelium-derived relaxing factors and endothelium-derived contracting factors regulate vascular tone and platelet function, and may constitute a chemical barrier against the toxic effects of Reactive Oxygen Species. These endothelial functions are important not only to the inflammatory response but also to circulatory regulation and to the potential development of atherosclerosis. During

inflammation, the expression of adhesion molecules on the endothelial surface facilitates leukocytes adherence and exudation (Hansen, 1998).

d. Platelets

Platelets contribute to the inflammatory response by several mechanisms. Platelets contain and release numerous inflammatory mediators, including fibrinogen, plasminogen and other components, including lipids and serotonin. Platelet-releasing mediators participate in the plasma protease systems. Several of these mediators released from platelets are direct activating agents for neutrophils. Conversely, neutrophil-releasing mediators serve to alter platelet function. Platelets interact with lymphocytes, providing the cell contact and reagents for prostaglandin synthesis. Platelets can also interact with fibroblasts, stimulating collagen and fibronectin synthesis during resolution (Rosenberg and Gallin, 2003).

Table 3. Endothelium-derived mediators (Hansen, 1998)

Endothelium-derived Relaxing Factors (Vasodilators)
Lipids
- Prostacyclin
Other Small Molecules
- Histamine
- Nitric oxide (Endothelium-derived Relaxing Factor)
- Endothelium-derived hyperpolarizing Factor
Endothelium-derived Contracting Factors (Vasoconstrictors)
Proteins
- Endothelin
- Angiotensin II
Lipids
- Endoperoxide
- Thromboxane A_2
Other Small Molecules
- Reactive Oxygen species
Other Endothelium-derived Mediators
Proteins
- Growth Factors
- Matrix proteins
- Coagulation factors
- Anticoagulation factors
- Fibrinolytic factors
- Antigens
- Enzymes
- Receptors
Other Small Molecules
- Adhesion molecules

A.2.2 Processes of Cellular Response

Cell components in the inflammatory system, including granulocytes, macrophages, endothelium and platelet work together to eliminate foreign materials. The cellular stages of acute inflammatory response are margination, emigration, chemotaxis and phagocytosis (Chandrasoma and Taylor, 1998).

a. Margination and Rolling of Leukocytes

In normal blood vessels, the cellular elements of blood are confined to a central axial stream which is separated from the endothelial surface by a zone of plasma. This separation depends on normal blood flow, which creates a physical force that tends to keep the heaviest cellular particles in the center of the vessels. As a result of the vascular response, leukocytes move to the periphery in contact with the endothelium (margination). Subsequently, leukocytes tumble on the endothelial surface, transiently sticking along the way (Rolling). In the next step, the weak and transient adhesions involved in rolling are mediated by the selectin family of adhesion molecules (Table 4). Selectins are receptors expressed on leukocytes and endothelium that contain an extracellular domain that binds sugar (lectin part). The three members of this family are E-selectin (CD62E), P-selectin (CD62P) and L-selectin (CD62L). Selectins bind sialylated oligosaccharides that are attached to mucin-like glycoprotein on various cells. They are up-regulated after stimulation by specific mediators such as histamine, IL-1 and TNF. Therefore, binding of leukocytes is largely restricted to endothelium at sites of infection or tissue injury where the mediators are produced (Chandrasoma and Taylor, 1998).

Table 4. Endothelial and leukocyte adhesion molecules (Kumar et al., 2007)

Endothelial Molecule	Leukocyte Molecules	Major Role
P-selectin	Sialyl-Lewis X-modified proteins	- Rolling
E-selectin	Sialyl-Lewis X-modified proteins	- Rolling and adhesion
GlyCam-1 (CD34)	L-selectin	- Rolling
ICAM-1	CD11/CD18 integrins (LFA-1, Mac-1)	- Adhesion, arrest and transmigration
VCAM-1	VLA-4 integrin	- Adhesion
CD31	CD31	- Transmigration

b. Adhesion and Transmigration

The next step in the reaction of leukocytes is firm adhesion to endothelial surfaces. This adhesion is mediated by integrins expressed on leukocyte cell surfaces interacting with their ligands on endothelial cells (Table 4). Integrins are transmembrane heterodimeric glycoproteins that function as cell receptors for extracellular matrix. Integrins are normally expressed on leukocyte plasma membranes in a low-affinity form and do not adhere to their appropriated ligands until the leukocytes are activated by chemokines. Chemokines are chemoattractant cytokines that are secreted by many cells at sites of inflammation and are displayed bound to proteoglycans on the endothelial surface. When the adherent leukocytes encounter the displayed chemokines, the cells are activated, and their integrins undergo conformational changes and cluster together, thus converting to a high-affinity form. At the same time, TNF and IL-1 activate endothelial cells to increase their expression of ligands for integrins. These ligands include ICAM-1 which binds to the integrins LFA-1 and Mac-1, and VCAM-1 which binds to the integrin VLA-4. The net result of cytokine-stimulated increased integrin affinity and increased expression of integrin ligands is stable attachment of leukocytes to endothelial cells at the site of inflammation. After leukocytes are arrested on the endothelial surface, they migrate through the vessel wall by penetrating the intercellular junctions of endothelium (diapedesis). Migration of leukocytes is driven by chemokines produced in extravascular tissue which stimulate movement of leukocytes toward their chemical gradient. In addition, PECAM-1, a cellular adhesion molecule expressed on leukocytes and endothelial cells, mediates the binding events needed for leukocytes to traverse the endothelium. After passing through the endothelium, leukocytes cross vascular basement membranes by focally degrading them with secreted collagenases (Chandrasoma and Taylor, 1998).

c. Chemotaxis

After diapedesis, leukocytes migrate toward the site of the tissue injury along the chemical gradient by a process called *chemotaxis*. Both exogenous and endogenous substances can be chemotactic for leukocytes, including (1) bacterial products, particularly peptides with *N*-formylmethionine termini; (2) cytokines, especially those of the chemokine family (Table 5); (3) components of complement system, particularly C5a;

and (4) products of the lipoxygenase pathway of arachidonic acid metabolism, particularly LTB_4. These mediators are produced in response to infections, tissue damage and immunological reactions. Leukocytes infiltration in all these situations results from the action of various combinations of mediators. The type of emigration of leukocyte varies with the age of the inflammatory response and with the type of stimulus. In most forms of acute inflammation, neutrophils predominate in the inflammatory filtrate during the first 6 to 24 hours and are replaced by monocytes within 24 to 48 hours. However, there are exceptions to this pattern of cellular exudation. In certain infections, such as *Pseudomonas* organisms, the cellular infiltrate is dominated by continuously recruited neutrophils for several days. In viral infections, lymphocytes may be the first cells to arrive. In some hypersensitivity reactions, eosinophils may be the main cell type (Kumar et al., 2007).

Table 5. Chemokines and chemokine receptors (Abbas and Lichtman, 2003)

Chemokines		Chemokine Receptors	Functions
Original Name	**Systemic Name**		
IL-8	CXCL8	CXCR1	- Neutrophil Recruitment
		CXCR2	
GCP-2	CXCL6	CXCR1	
NAP-2	CXCL7	CXCR2	
ENA78	CXCL5		
Gro-α	CXCL1		
Gro-β	CXCL2		
Gro-γ	CXCL3		
IP-10	CXCL10	CXCR3	- T cell Recruitment to DTH sites
MIG	CXCL9		
I-TAC	CXCL11		
SDF-1	CXCL12	CXCR4	- Mixed leukocyte Recruitment
			- HIV co-receptor
BCA-1	CXCL13	CXCR5	- Lymphocyte Migration into follicles
RANTES	CCL5	CCR1	- Mixed leukocyte Recruitment
MIP-1α	CCL3	CCR5	
MCP-3	CCL7	CCR1	
		CCR2	
MCP-1	CCL2	CCR2	
MCP-2	CCL8		
MCP-4	CCL13	CCR2	
		CCR3	
Eotaxin	CCL11	CCR3	- Eosinophil and Basophil Recruitment

Table 5. Chemokines and chemokine receptor (cont.)

Chemokines		Chemokine Receptors	Functions
Original Name	**Systemic Name**		
TARC	CCL17	CCR4	- T cell and Basophil Recruitment
MDC	CCL22		
MIP-1β	CCK4	CCR5	- T cell, Dendritic cell, Monocytes and NK cell Recruitment - HIV co-receptor
MIP-3α	CCL20	CCR6	- Lymphocytes and Dendritic cell migration
ELC	CCL19	CCR7	- Lymphocytes and Dendritic cell migration into T cell zone of lymph node
SLC	CCL21		
I-309	CCL1	CCR8	- Lymphocyte Traffic
MEC	CCL28	CCR3 CCR10	- Dermal cell Migration
CTACK	CCL27	CCR10	
TECK	CCL25	CCR11	- Astrocyte Migration

d. Phagocytosis

Phagocytosis consists of three distinct but interrelated steps, including (1) recognition and attachment of the particle to the ingesting leukocyte, (2) engulfment, with subsequent formation of a phagocytic vacuole and (3) killing and degradation of the ingested material (Chandrasoma and Taylor, 1998; Kumar et al., 2007).

The first step in phagocytosis is recognition of the injurious agent by phagocytic cell, either directly binding to specific surface receptor or after the agent has been coated with opsonins. The later process is called opsonization. The most important opsonins are antibodies (IgG class), breakdown products of the complement protein C3 and plasma carbohydrate-binding lectins (Collectin). These opsonins are either present in the blood ready to coat microbes or produced in response to microbes. Leukocytes express receptors for opsonins that facilitate rapid phagocytosis of the coated microbes, including FcγRI for an Fc part of IgG, complement receptor 1 and 3 (CR1 and 3) for complement fragments, and C1q for the collectins (Chandrasoma and Taylor, 1998; Kumar et al., 2007).

Once recognized by leukocytes, a foreign particle is engulfed by the phagocytic cell, especially neutrophils and macrophages. Pseudopods are extended around the object, eventually forming a phagocytic vacuole (phagosome). IgG binding to FcR and binding of complement products to C3 receptors induces cellular activation that enhances the production of digesting lysosomes. The membrane of the vacuole then fuses with the membrane of a lysosomal granule, resulting in discharge of the granule's contents into the phagolysosome (Chandrasoma and Taylor, 1998; Kumar et al., 2007).

The last step of phagocytosis is the killing and degradation of microbes. When the offending agent is a microorganism, it must be killed before degradation occurs. The key steps in this reaction are the production of microbicidal substances within lysosome and fusion of lysosomes with phagosome, thus selectively exposing the ingested particles to the destructive mechanisms of the leukocytes. The most important microbicidal substances are reactive oxygen species (ROS) and lysosomal enzymes. Phagocytosis induces an oxidative burst (increase in oxygen consumption, glycogenolysis, glucose oxidation and production of ROS) that generates ROS due to the leukocyte NADPH oxidase (phagocytic oxidase). The azurophilic granules of neutrophil contain the enzyme myeloperoxidase that generates hypochlorous radical, a powerful oxidant and antimicrobial agent. In addition,

nitric oxide is produced and interacts with ROS to generate the higher effective antimicrobial substances such as peroxynitrite. The dead microorganism or necrotic tissues are then degraded by the action of lysosomal acid hydrolase (Chandrasoma and Taylor, 1998; Kumar et al., 2007).

A.3 Mediator of Acute Inflammatory Response

Both vascular and cellular responses of the inflammatory process just described are initiated, regulated and ultimately eliminated by the actions of numerous pro-inflammatory mediators (Table 6). Some of these mediators are stored within cells in inactive form and are activated by products of acute inflammation (Kumar et al., 2007).

A.3.1 The Plasma Protease

Among the central components of the inflammatory response are the three interacting groups of plasma proteases. The plasma proteases are mostly produced by the liver. Through the actions of plasma proteins as they convert each other from inactive to active forms, many major soluble mediators of inflammation are generated (Kumar et al., 2007).

a. The Coagulation Cascade and the Kinin System

A central event in the generation of several circulating mediators of inflammation is activation of Hageman factor (Fig. 2). Hageman factor (factor XII) is the intrinsic coagulation cascade that is synthesized by the liver and released in an inactive form in blood circulation. This protein is activated by encountering collagen, basement membrane or activated platelet at the site of endothelial injury. In the presence of high-molecular weigh kininogen (HMWK) cofactor, factor XII conformational changes to become factor XIIA. Activated Hageman factor (factor XIIa) initiates four systems involved in the inflammatory responses. In the clotting system, the factor XIIa-driven proteolytic cascade leads to activation of thrombin which cleaves circulating soluble fibrinogen to generate an insoluble fibrin clot. Factor Xa, which is an intermediate in the clotting cascade, causes increased vascular permeability and leukocyte emigration. The binding of thrombin to

protease-activated receptors (PARs) on endothelial cells leads to enhance leukocyte adhesion. Thrombin generates fibrinopeptides that increase vascular permeability and are chemotactic for leukocytes. Kinin system activation leads to the formation of bradykinin from its circulating precursor, HMWK. Bradykinin causes pain, and increases vascular permeability, arteriolar dilation and bronchial smooth muscle contraction. Kallikrein, an intermediary in kinin cascade, exerts chemotactic activity. Linked with kinin cascade, plasminogen activator and Kallikrein cleave plasminogen to plasmin that lead to cleave fibrin to form fibrin degradation products. The increase of fibrin-split products increases vascular permeability while plasmin can cleave the C3 complement protein, resulting in production of C3a which is discussed later (Kumar et al., 2007).

b. The Complement System

The complement is a primary mediator of the humoral immune response that enables the body to produce an inflammatory response, lyse foreign cells and increases phagocytosis. Proteins of the complement system are produced by the liver. The complement system consists of a group of proteins that are normally present in blood circulation as functionally inactive precursors. When a complement reaction occurs, the inactive complement proteins are converted to their active form in a sequence of reactions as shown in Fig. 3. The modified or split complement protein such as C3b, C3a and C5a that are released during activation function in the next step of the pathway or are released into the tissue fluid to produce biologic effects important in inflammation. First, complement has been shown to mediate the lytic destruction of many kinds of cells, including red blood cells, platelets, bacteria and lymphocytes. All complement pathways induce cytolysis. Second, a major biologic function of complement activation is opsonization (C3b, a C3 product) such that pathogens are engulfed and cleared more efficiently by macrophages. Third, chemotactic complement products (C3a and C5a) trigger an influx of leukocytes. These white blood cells remain fixed in the area of complement activation through attachment to specific sites on C3b and C4b molecules. Fourth, production of anaphylatoxin (C3a and C5a) induces histamine release from mast cells, leading to contraction of smooth muscle, increased vascular permeability and edema (Kumar et al., 2007).

Table 6. Role of mediators in different reactions of inflammation (Kumar et al., 2007)

Inflammatory Responses	Inflammatory Mediators
Vasodilation	- Prostaglandins
	- Nitric oxide
	- Histamine
Increased Vascular Permeability	- Histamine
	- Serotonin
	- C3a and C5a (liberating vasoactive amines from mast cells and other cells)
	- Bradykinin
	- Leukotrienes C_4, D_4, E_4
	- Platelet-activating factor (PAF)
	- Substance P
Leukocyte Recruitment and Activation	- Tumor Necrosis Factor
	- Interleukine-1
	- Chemokines
	- C3a and C5a
	- Leukotrienes B_4
Fever	- Tumor Necrosis Factor
	- Interleukine-1
	- Prostaglandins
Pain	- Prostaglandins
	- Bradykinin
	- Neuropeptides
Tissue Damages	- Lysosomal enzymes of leukocytes
	- Reactive Oxygen Species
	- Nitric oxide

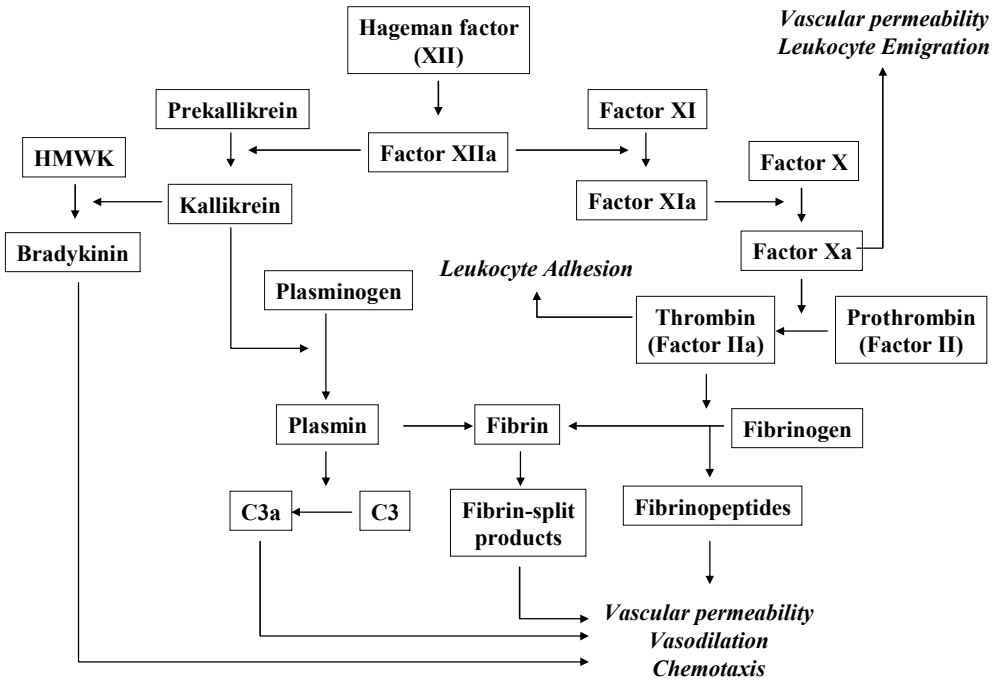

Figure 2. Interrelationships among the coagulation cascade, the fibrinolytic system, the complement system and the kinin System triggered by activation of factor XII (Kumar et al., 2007)

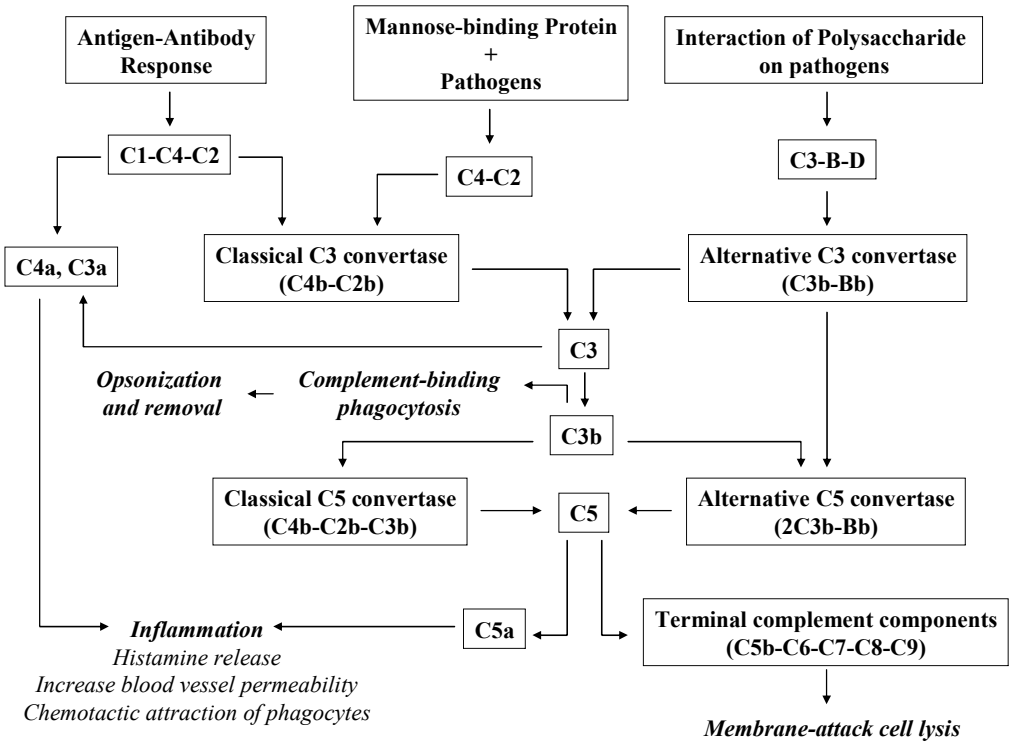

Figure 3. The complement cascade

Complement proteins circulate in inactive form until activation by antigen-antibody complex (Classical pathway), polysaccharide molecule on bacterial surface (Alternative pathway) and mannose-containing molecules on surface of pathogen (Lectin pathway). Activation of one protein causes sequential activation of another, enhancing the inflammatory response and leading to formation of a membrane attack complex (MAC). This complex destroys invading microorganisms by perforating their walls (Sommer, 2002).

A.3.2 Lipid Mediators

Lipid mediators are a complex group of chemicals that participate in augmenting the inflammatory response. This group includes the prostaglandins, leukotrienes and platelet-activating factors. The first two groups are the metabolites of arachidonic acid by cyclooxygenases (Rosenberg and Gallin, 2003).

a. Arachidonic Acid Metabolites

Arachidonic acid (AA) is a 20-carbon polyunsaturated fatty acid (within four double bonds) derived from dietary linoleic acid and present in the body mainly in its esterified form as a component of cell membrane phospholipids. AA is released from the cell membrane in response to cellular phospholipases that can be stimulated by chemical, mechanical or physical stimuli, or by inflammatory mediators such as C5a. AA metabolism proceeds along one of two enzymatic pathways (Fig. 4); (1) Cyclooxygenases stimulate the synthesis of prostaglandins and thromboxanes; and (2) Lipoxygenase is responsible for production of leukotrienes and lipoxin. The activities of AA metabolites are shown in Table 7. Prostaglandins arise from the cyclooxygenases pathway and their activities are vasodilation, increased permeability, enhancing the sensitivity of pain ending and inducing platelet aggregation. In case of leukotrienes, LTB_4 is a potent chemotactic agent that causes leukocyte aggregation while LTC_4, LTD_4 and LTE_4 cause smooth muscle contractility, bronchospasm and increased vascular permeability (Rosenberg and Gallin, 2003; Hansen, 1998; Kumar et al., 2007).

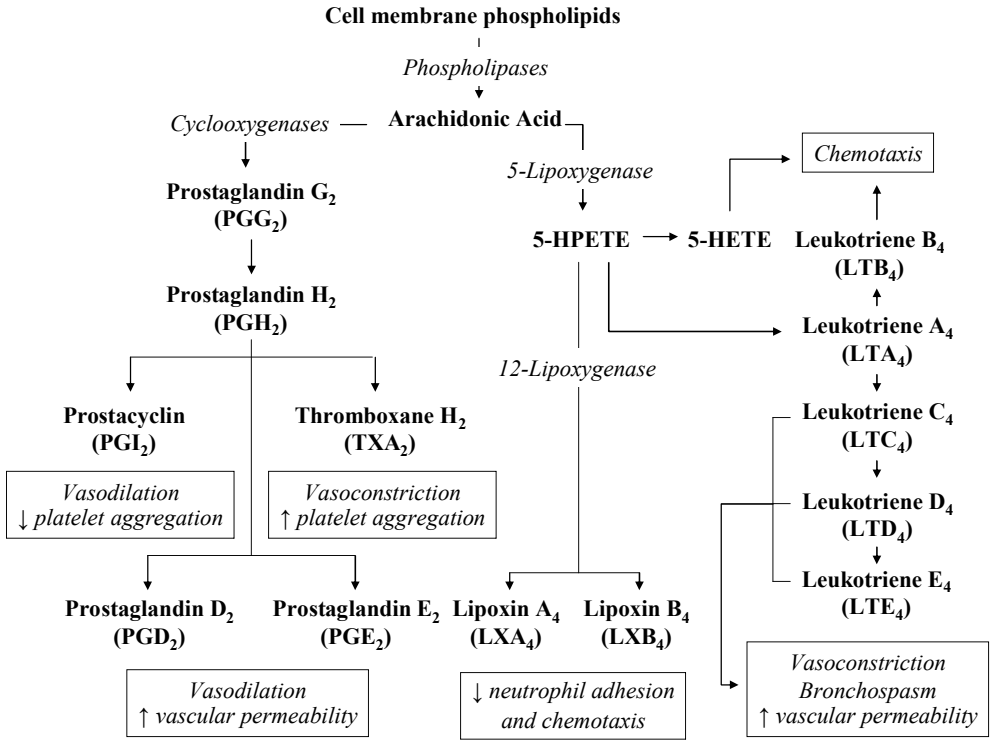

Figure 4. Arachidonic acid pathways

Lipid mediators of inflammation include the products of the fatty acid, arachidonic acid, released from the cell membrane by enzyme phospholipases. Arachidonic acid may be metabolized by either cyclooxygenases (COX-1 or COX-2) or lipoxygenases (5-lipoxygenase or 12-lipoxygenase). 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid (Hansen, 1998; Kumar et al., 2007)

Table 7. Principal inflammatory action of arachidonic acid metabolites (Kumar et al., 2007)

Action	Arachidonic Acid Metabolites
Vasodilation	- Prostaglandin E_1 (PGE_1) - Prostaglandin E_2 (PGE_2) - Prostaglandin D_2 (PGD_2)
Vasoconstriction	- Thromboxane A_2 - Leukotriene C_4 (LTC_4) - Leukotriene D_4 (LTD_4) - Leukotriene E_4 (LTE_4)
Increased vascular permeability	- Leukotriene C_4 (LTC_4) - Leukotriene D_4 (LTD_4) - Leukotriene E_4 (LTE_4)
Chemotaxis and Leukocyte adhesion	- Leukotriene B_4 (LTB_4)

b. Platelet-Activating Factor

Originally named for its ability to aggregate platelets and cause degranulation, platelet-activating factors (PAF) is another phospholipid-derived mediator with a broad spectrum of inflammatory effects. PAF is acetyl glycerol ether phosphocholine that is generated from the membrane phospholipids of neutrophils, monocytes, basophils, endothelial cells and platelets by the action of phospholipases A_2. PAF causes vasoconstriction and bronchoconstriction which are found in immediate hypersensitivity reactions. Vasodilation and increased vascular permeability by PAF is more potent than histamine. PAF can elicit most of the reaction of inflammation, including enhanced leukocyte adhesion, chemotaxis, leukocyte degranulation and the oxidative burst; it can stimulate the synthesis of eicosanoids (Rosenberg and Gallin, 2003; Hansen, 1998; Kumar et al., 2007).

A.3.3 Peptide and Amines

a. Vasoactive Amines

Histamine and serotonin are vasoactive amines that are primarily released from mast cell, circulating basophil and platelet during acute inflammation. Performed histamine is released from mast cell granules in response to a variety of stimuli: (1) physical injury such as trauma or heat; (2) immune reactions involving binding of IgE antibodies to Fc receptors on mast cells; (3) C3a and C5a fragment of complement (anaphylatoxins); (4) leukocyte-derived histamine-releasing proteins; (5) substance P of neuropeptides; and (6) cytokines such as IL-1 and IL-8. The functions of histamine binding to Histamine receptors (H1, H2 or H3) cause arteriolar dilation, increased vascular permeability, and induce venular endothelial contractions. Serotonin (5-hydroxytryptamine) is a performed vasoactive mediator with effects similar to those of histamine. Serotonin is found within platelet dens body granules and is released during platelet aggregation (Chandrasoma and Taylor, 1998; Rosenberg and Gallin, 2003; Kumar et al., 2007).

b. Neuropeptides

Neuropeptides released from neurons in response to local tissue damage are one of the aspects of the connection between the nervous system and inflammatory response. This group of mediators includes substance P, vasoactive intestinal peptide, and somatostatin and calcitonin gene-related peptide. The neuropeptides transmit pain signals, regulated vessel tone and modulate vascular permeability. Nerve fibers that secrete neuropeptides are prominent in the lung and gastrointestinal tract (Chandrasoma and Taylor, 1998; Rosenberg and Gallin, 2003).

A.3.4 Cytokines

Cytokines are polypeptide products of many cell types that function as mediators of inflammation and immune responses (Table 8). In acute inflammation, the major cytokines are TNF and IL-1, whereas IFN-γ and IL-12 are more important in chronic inflammation. TNF and IL-1 are mainly produced by activated macrophages as well as mast cells, endothelial cells and some other cell types (Fig. 5). TNF and IL-1 stimulate the expression of adhesion molecules on endothelial cells, resulting in increased leukocyte binding and recruitment, and enhance the production of chemokines and eicosanoids. TNF also increases the thrombogenicity of endothelial cells and causes aggregation and activation of neutrophils. Il-1 activates tissue fibroblasts, resulting in increased proliferation and production of extracellular matrix (ECM). In the systemic acute-phase reaction, TNF and IL-1 work together with IL-6 to induce hepatic synthesis of various acute-phase proteins, metabolic wasting, neutrophil release into the circulation, and release of adrenocorticotropic hormone (inducing corticosteroid synthesis and release). In addition, these cytokines affect the thermoregulatory center in the hypothalamus to produce fever. IL-1 and other cytokines induce an increase in the number and immaturity of circulating neutrophils by stimulating their production in the bone marrow (Tables 2 and 8). Furthermore, lethargy and sleep result from the effects of IL-1 and TNF on the central nervous system. In contrast, IL-4, IL-10 and TGF-β exert a positive feedback to the adaptive immune response and limit the inflammation by their inhibitory effects (also called anti-inflammatory cytokines) (Kumar et al., 2007).

Table 8. Cytokines and their functions (Peterson et al., 1995)

Cytokines	Origins	Functions
Interferon-α (IFN-α)	- Leukocytes	- Inhibits virus replication - Toxic to cancer cells - Stimulates leukocytes - Facilitates killer cell activity - Produces fever - Increases B- and T-cell activity
Interferon-β (IFN-β)	- Fibroblasts	- Inhibits virus replication - Toxic to cancer cells - Facilitates killer cell activity - Produces fever
Interferon-γ (IFN-γ)	- Activated T-cells	- Inhibits virus replication - Promotes antigen expression - Activates macrophages - Inhibits cell growth - Induces myeloid cell lines
Interleukin-1 (IL-1)	- Mononuclear phagocytes	- Stimulates T-cells and macrophages - Induces acute-phase reaction of inflammation - Induces IL-2 secretion, fever production - Similar to TNF and endogenous pyrogens
Interleukin-2 (IL-2)	- T-helper cells	- Promotes growth of T-cells - Enhances functions of natural killer (NK) cells - Assists T-cell maturation in thymus

Table 8. Cytokines and their functions (cont.)

Cytokines	Origins	Functions
Interleukin-3 (IL-3)	- T-cells - Endothelial cells - Fibroblasts	- Induces proliferation and differentiation of other lymphocytes pluripotential stem cells, mast cells
Interleukin-4 (IL-4)	- Lymphocytes	- Promotes T-cell/B-cell interation - Promotes synthesis of IgE by B-cells and T-cell growth - Promotes mast cell and hematopoietic cell growth
Interleukin-5 (IL-5)	- T-cells	- Promotes the growth and differentiation of B-cell to secret IgA - Induces differentiation of eosinophils
Interlkin-6 (IL-6)	- Mononuclear macrophages - T-cell - Tumors - Nonlymphoid cells	- Promotes immunoglobulin secretion by B-cells - Induces fever - Promotes release of inflammation factor from the liver - Promotes differentiation of hematopoietic stem cells and never cells
Tumor necrosis factor (TNF)	- Monocytes - Macrophages	- Induces leukocytosis, fever, weight loss, inflammation, necrosis of some tumors - Stimulates lymphokines synthesis - Activates macrophages - Toxic to viruses and tumor cells

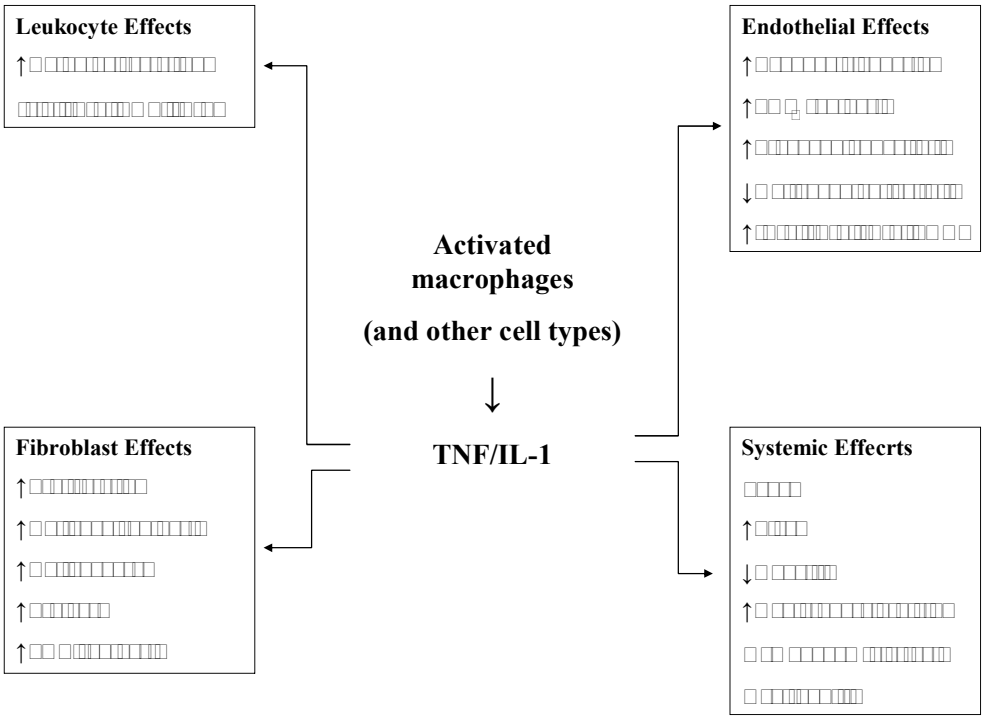

Figure 5. Major effects of TNF and IL-1 in inflammation (Kumar et al., 2007)

A.3.5 Acute-Phase Reactants

Along with the cellular response, a group of systemic effects, called the acute-phase response, occurs. The acute-phase response, which is initiated within hours or day of inflammation or infection onset, includes changes in the concentrations of plasma protein, increased erythrocyte sedimentation rate (ESR), fever, increased numbers of leukocytes, skeletal muscle catabolism, and negative nitrogen balance. These responses are generated after the release of the cytokines, IL-1, TNF and IL-6. These cytokines affect the production of acute-phase reactants that are produced from the liver (Table 9). C-reactive protein, serum amyloid A and fibrinogen are among the best characterized of acute-phase reactants which induce quantities in response to inflammatory stimuli. C-reactive protein has received attention as the marker for the inflammation associated with advanced coronary artery disease. Similarly, serum amyloid A has been shown to induce cellular chemotaxis and adhesion. In addition to its role in hemostasis, fibrinogen has been shown to activate stimulated neutrophils to produce the pro-inflammatory cytokine IL-8 and to stimulate chemokine secretion from macrophages through the Toll-like receptor 4 (TLR4) (Rosenberg and Gallin, 2003; Sommer, 2002).

Table 9. Acute-phase reactants (Hansen, 1998)

Reactants	Significant Functions
C-reactive Protein	- Binds to bacterial proteins - Initiating complement activation by the alternative pathway
Fibrinogen	- Involved in barrier formation, clotting and tissue preparation for healing
α_1-Antitrypsin	- Inhibits tissue breakdown by protease enzymes
α_2-Macroglobulin	- Inhibits tissue breakdown by protease enzymes
Fibronectin	- Involved in cellular attachment - Chemotactic factor (attracts neutrophils)
Ceruloplasmin	- Inhibits reactive oxygen species
Haptoglobin	- Accelerates wound healing

A.3.6 Free Radicals

Free radicals are defined as molecules having an unpaired electron in their outer orbit, by which they are unstable and vary reactive. Free radicals play an important role in the human biological processes such as vascular systems, neurological system and immune system, especially in the inflammatory processes. Free radicals are generally classified into two types, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). In inflammatory conditions, ROS and RNS are normally generated to act as toxic agents that participate in phagocytosis (Fang et al., 2002).

a. Reactive Oxygen Species (ROS)

During inflammatory processes, ROS are synthesized by the NADPH oxidase (phagocytic oxidase) pathway (Fig. 6) and are released from neutrophils and macrophages, in the activation of microbes, immune complexes, cytokines and other inflammatory stimuli. When ROS are produced within lysosomes, they function to destroy phagocytosed microbes and necrotic cells. In low levels of secretion, ROS can increase chemokine, cytokine and adhesion molecule expression, thus amplifying the cascade of inflammatory mediators. At higher levels, these mediators are responsible for tissue injury through several mechanisms, including (1) endothelial damage, with thrombosis and increased microvascular permeability; (2) protease activation and anti-protease inactivation, with a net increase in breakdown of ECM; (3) direct injury to other cell types (e.g. tumor cells, erythrocytes and parenchymal cells); (4) up-regulation of adhesion molecules such as ICAM-1 and P-selectin; (5) autocatalytic destruction of neurotransmitters and hormones; (6) lipid peroxidation and oxidation stress; (7) DNA damages; and (8) activation of PARP [Poly(ADP-ribose) polymerase] that participates in both acute and chronic inflammation (Salvemini et al., 2006; Kumar et al., 2007).

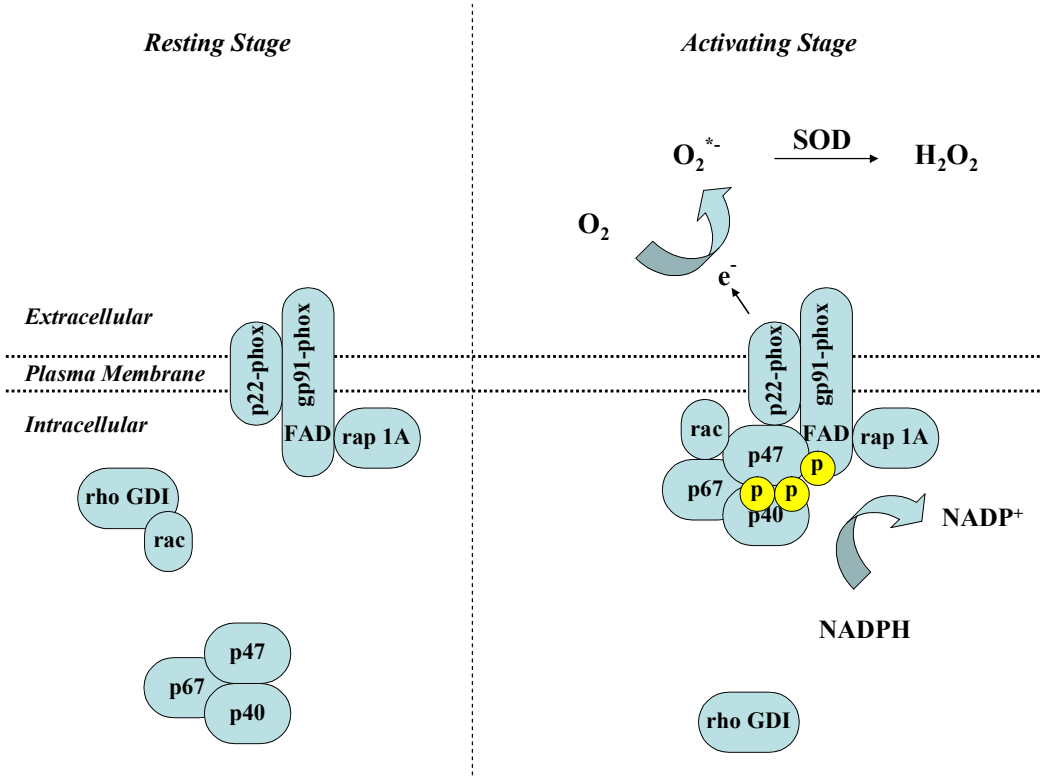

Figure 6. The protein components of the phagocytic NADPH oxidase

Upon activation, the cytoplasmic components Rac, p67-phox, p47-phox and p40-phox are translocated to the cell membrane to form the catalytic complex. Once formed, the complex can catalyze the conversion of moleculear oxygen (O_2) to superoxide (O_2^{*-}) which is converted to the toxic oxygen metabolite hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD). The proteins associate specific SH3 domain recognition sites (Rosenberg and Gallin, 2003).

b. Reactive Nitrogen Species (RNS)

Nitric oxide is a short-lived, soluble, free radical gas produced by many cell types and capable of mediating a variety of functions. Nitric oxide is synthesized de novo from L-arginine, oxygen and NADPH by the enzyme nitric oxide synthase (NOS) as seen in Fig. 7. There are three isoforms of NOS with different tissue distribution (Fig. 8). Type II NOS or iNOS is induced by a number of inflammatory stimuli, most notably by IL-1, TNF IFN-γ and bacterial endotoxin, and is responsible for production of nitric oxide in inflammatory reactions. iNOS can be presented in many cell types, including monocyte/macrophages, endothelial cells, hepatocytes, cardiac myocytes and respiratory epithelium. The major roles of nitric oxide are (1) relaxation of vascular smooth muscle (vasodilation), (2) antagonism in all stages of platelet activation (adhesion, aggregation and degranulation), (3) reduction of leukocyte recruitment at inflammatory sites and (4) action as microbicidal (cytotoxic) agent in activated macrophages. In addition, nitric oxide and its metabolites can enhance the activity and the expression of COX enzymes that initiate the production of arachidonic metabolites, leading to a cascade of inflammatory responses as described above (Mollace et al., 2005).

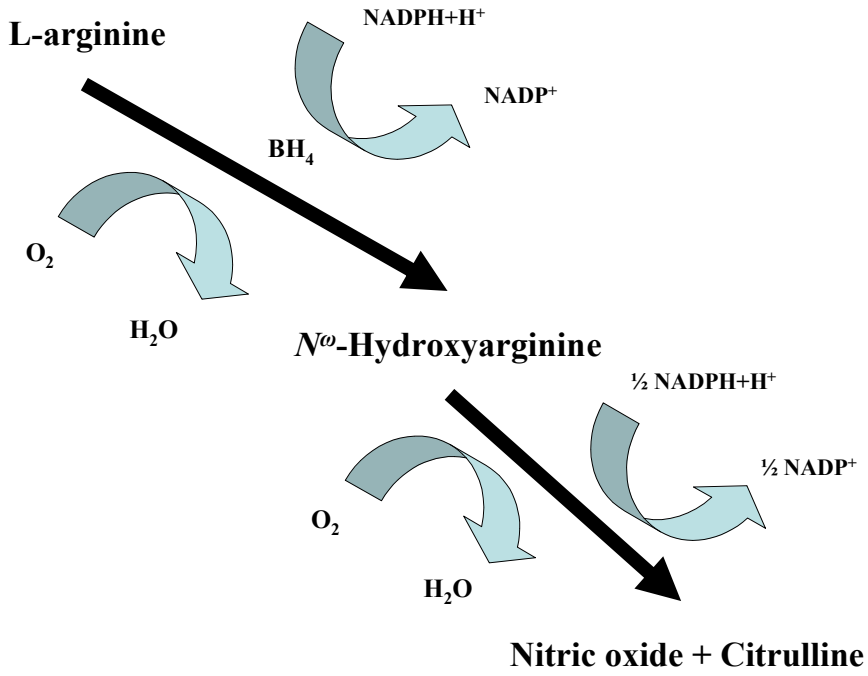

Figure 7. The nitric oxide synthase reaction

Nitric oxide synthesis from arginine is a reaction which involves two separate mono-oxygenation steps. In the first step, N^{ω}-Hydroxyarginine is an intermediate species formed by a reaction requiring one O_2 and one NADPH and the presence of tetrahydrobiobterin (BH_4). The second step in nitric oxide synthase reaction results in the oxidation of N^{ω}-Hydroxyarginine to form citrulline and nitric oxide (Knowles and Moncada, 1994).

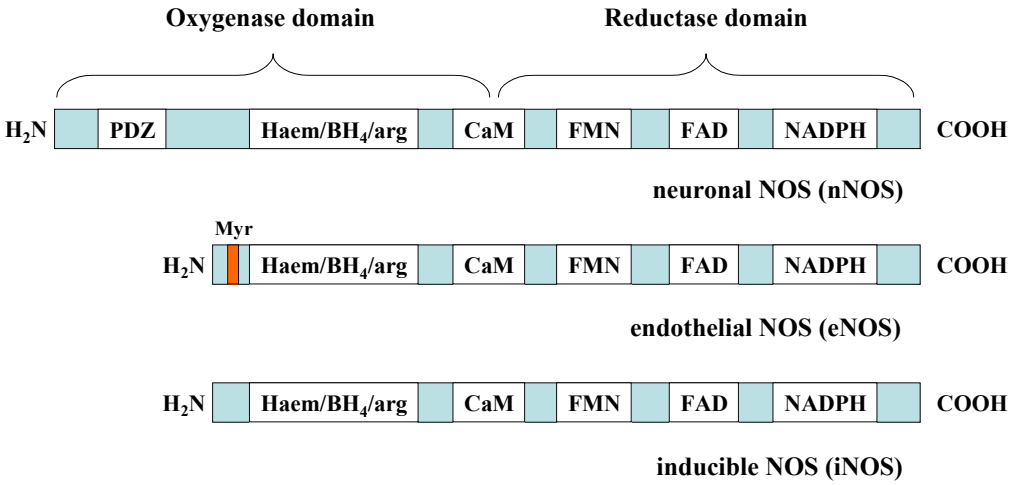

Figure 8. Schematic representation of nitric oxide synthase (NOS) isoforms

Type I (nNOS) is constitutively expressed in neurons. Type II (iNOS) is an inducible enzyme present in macrophages and endothelial cells. Type III (eNOS) is a constitutively synthesized found within endothelium. PDZ, PDZ domain (GLGF repeats); CaM, calmodulin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Myr, the N-terminal myristylation site (Knowles and Moncada, 1994; Hobbs et al., 1999).

A.4 The Outcome of Acute Inflammation

The consequences and severity of acute inflammation are modified by the nature of stimuli, the affected site and tissue, the intensity of injury, and the ability of host to mount a response. However, the outcome of acute response is either resolution and healing or chronic phase progression. The resolution of acute responses occurs when the injury is limited or short lived, when there has been no or minimal tissue damage, and when the tissue is capable of replacing any irreversible injured cells. These reactions occur via the release of anti-inflammatory mediators such as IL-4, TGF-β, IL-10 and IL-13, which mostly reduce the inflammatory responses via the suppression of inflammatory mediator expression, neutralization of degradation enzymes, normalization of vascular permeability, cessation of leukocyte emigration and induction of the apoptosis of inflammatory cells. After phagocytic cells remove all derbis, new tissues and their functions are regenerated from surrounding fibroblast, epithelial cells and endothelial cells. Numerous soluble mediators, including epidermal growth factor, keratinocyte growth factor, platelet-derived growth factor, fibroblast growth factors, TGF-α and TGF-β promote these events. If tissue regeneration does not occur, dead tissues are replaced with nonfunctional scar tissue. In contrast, the progression to chronic inflammation may follow acute inflammation if the offending agent is not removed. This event is present when the exudative phase of inflammation lasts longer than 2 weeks. Inflammation may persist indefinitely owing to virulence of the invading organism, the severity of trauma, or the inadequacy of the patient's immune system (Hansen, 1998; Kumar et al., 2007; Rosenberg and Gallin, 2003).

B. Chronic Inflammatory Responses

When acute inflammation persists, it becomes chronic inflammation either through incomplete clearance of the initial inflammatory focus or as a result of multiple acute events occurring in the same location. Agents that evoke chronic inflammation are typically low-grade, persistent irritants that are unable to penetrate deeply or spread rapidly. Among the cause of chronic inflammation are foreign bodies such as talc, silica, asbestos and surgical suture materials. Many viruses provoke chronic inflammatory responses as do

certain bacteria, fungi and larger parasites of moderate to low virulence. Examples are the tubercle bacillus, the treponema of syphilis and the actinomyces (Sommer, 2002).

In contrast to acute inflammation, the characteristic of chronic inflammation is the accumulation of macrophages and lymphocytes instead of influx of neutrophils. Macrophages that migrate to the sites of acute inflammation are transformed to the epitheloid cells (activated macrophages) by bacterial endotoxin, microbial products, cytokines secreted by T-cells and acute inflammatory mediators. After activation, macrophages release a wide variety of biologically active products that, if uncontrollable, can result in tissue injury and fibrosis that are characteristic of chronic inflammation. The example of macrophages mediators are protease, ROS and RNS, lipid mediators and pro-inflammatory cytokines (IL-1, TNF). In addition, lymphocytes migrate into the inflammatory site in response to chemokine secretion. Lymphocyte and macrophages interact in a bidirectional way (Fig. 9) which is an important role of chronic inflammatory responses. Macrophages display antigens to T-cells, express membrane molecules (co-stimulators) and produce cytokine (notably IL-12). Activated T-cells, in turn, produce IFN-γ which is the potent activator of macrophages. The activated B lymphocytes respond to the antigen presentation by macrophages produced antibodies directed either against persistent antigens in the inflammatory site or against altered tissue components. Moreover, the chronic response involves the proliferation of fibroblast instead of exudates. As a result, the risk of scarring and deformity is considered greater than acute inflammation. The two patterns of chronic inflammation are a nonspecific chronic inflammation and granulomatous inflammation (Kumar et al., 2007).

Nonspecific chronic inflammation involves a diffuse accumulation of macrophages and lymphocytes at the site of injury. Ongoing chemotaxis causes macrophages to infiltrate the inflammatory sites where they accumulate owing to prolonged survival and immobilization. In addition, sensitized lymphocytes and plasma cells are also emigrated in response to chemotaxis. These cells are scattered diffusely throughout the tissue. These mechanisms lead to fibroblast proliferation with subsequent scar formation which replaces the normal connective tissue or the functional parenchymal tissues of the involved structures. For example, scar tissue resulting from chronic inflammation of the bowel causes narrowing of the bowel lumen (Sommer, 2002).

A granulomatous lesion results from chronic inflammation. A granuloma typically is a small, 1 to 2-mm lesion in which there is a massing of macrophages

surrounded by lymphocytes. These modified macrophages to epitheloid cells are derived from blood monocytes. Granulomas can form in the setting of persistent T-cell responses to certain microbes where T-cell-derived cytokines are responsible for chronic macrophage activation. Granulomatous inflammation is associated with foreign bodies such as splinters, sutures, silica and asbestos, and with microorganisms that cause tuberculosis, syphilis, sarcoidosis, deep fungal infection and brucellosis. These types of agents are commonly poorly digested and are not easily controlled by other inflammatory mechanisms. The epitheloid cells in granulomatous inflammations may clump in a mass (granuloma) or coalesce, forming a large, multinucleated giant cell that attempts to surround the foreign agent. A dense membrane of connective tissue eventually encapsulates the lesion and isolates it. However, granuloma formation does not lead to eradiation of the causal agent which is resistant to killing or degradation, and granulomatous inflammation with subsequent fibrosis may even be the major cause of organ dysfunction in some diseases such as tuberculosis (Chandrasoma and Taylor, 1998; Kumar et al., 2007; Sommer, 2002).

The containment and destruction of the agent are largely depend on immunologic reactivity, whether these are achieved by (1) direct killing by activated lymphocytes, (2) interaction with antibodies produced by plasma cells, or (3) activation of macrophages by lymphokines produced by T lymphocytes. With the exception of foreign reactions, chronic inflammation is associated with tissue necrosis and implies serious clinical illness such as liver failure in chronic hepatitis. Chronic inflammation is a feature of many chronic diseases that are characterized either by total lack of recovery or by a long recovery period. In certain situations, fibrous scarring resulting from a repair mechanism can cause diseases. For example, fibrosis of the pericardial sac in chronic pericarditis restricts cardiac filling and causes heart failure, and pulmonary fibrosis causes respiratory failure. When removal and neutralization of the injurious agent is ultimately achieved, the tissue heals, usually by fibrosis. The chronic inflammatory cells disappear, and an acellular fibrous scar marks the site of injury (Chandrasoma and Taylor, 1998; Kumar et al., 2007; Sommer, 2002).

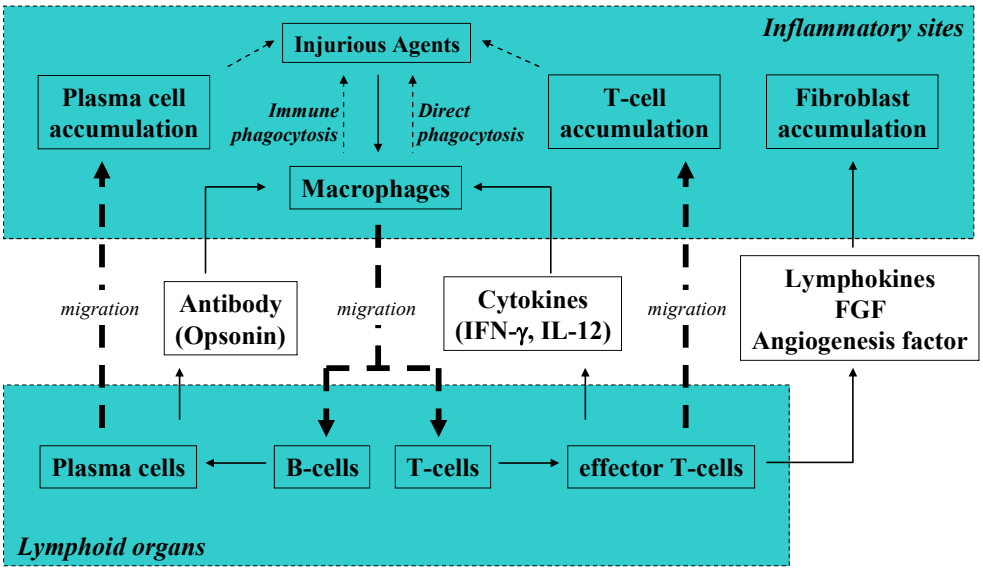

Figure 9. Mechanisms of chronic inflammatory responses

Cellular components are seen as part of the immune response. In most cases, the persistent injurious agents are antigenic and lead to an immune response involving T cells, B cells and macrophages. Foreign body granuloma formation appears to be a direct phagocytic response to inert material and the immune response is not involved. FGF, fibroblast growth factors (Chandrasoma and Taylor, 1998).

1.2.2.5 Anti-Inflammatory Drugs

Commonly used anti-inflammatory drugs can be classified into four group as shown in Table 10 (Hansen, 1998). Steroids such as cortisone interrupt the formation of arachidonic acid from membrane phospholipid, suppressing the production of prostaglandins and leukotrienes. Glucocorticoids relieve the pain and swelling of inflammation but introduce the risk of damage and metabolic side effects. The other group is called non-steroidal anti-inflammatory drugs (NSAID) including aspirin, ibuprofen and naproxen. This group interrupts prostaglandin synthesis by inhibiting COX enzyme activity in arachidonic metabolite pathways. NSAID have fewer side effects than cortisol, but may have less capacity to relieve symptoms. Agents such as gold salts may be used in an effort to induce remission in chronic inflammation conditions such as arthritis by unknown mechanisms. Cytotoxic agents such as methotrexate have been employed in some disorders. When inflammation is initiated by an immune mechanism, immunosuppressive agents or antihistamines may be used. In the infection-induced inflammation, antibiotic therapy may be used as bactericidal agents (Hansen, 1998).

Table 10. Anti-inflammatory drugs in common use (Hansen, 1998)

Anti-Inflammatory Class	Examples	Actions
Group I agents Salicylates and NSAID	Aspirin Ibuprofen Naproxen	- Inhibition of COX enzymes - Decreasing prostaglandin synthesis
Group II agents Remission-inducing drugs	Gold salts Penicillamine Hydroxychloroquinone	- Inhibition of phagocytosis - Inhibition of lysosomal enzymes - Decrease in immunoglobulin
Group III agents Glucocorticoids	Cortisol Cortisone Hydrocortisone Prednisone	- Inhibition of arachidonic formation
Group IV agents Cytotoxic agents	Cyclophosphamide Methotrexate	- Inhibition of lymphocyte activity

1.2.3 Signaling Transduction of LPS-induced TLR4 in Macrophages

1.2.3.1 Role of Macrophages in Inflammation

Macrophages represent a family of mononuclear leukocytes that are widely distributed throughout the body, within and outside lympho-hemopoietic organs. Depending on their origin and local environment, macrophages vary considerably in lifespan and phenotype. The functions of macrophages within tissues are homeostatic, regulating the local and systemic milieu through diverse plasma membrane receptors and varied secreting products. The activation of macrophages generates signals that promote growth, differentiation and death of other cells, recognizing and engulfing senescent and abnormal cells. These activities contribute substantially to recognition and defense functions against invading microorganisms, foreigner particulates, and other immunogens. In inflammatory processes, macrophages' functions complement those activities of neutrophils during acute response and take on a more central role during chronic inflammation (Gordon, 2003).

In the acute phase response, monocytes in blood circulation are recruited to the site of inflammation by the secretion of chemotaxis factors from neutrophils, and then mature into macrophages in response to inflammatory stimuli. Because of their longer lifespan which is 3-4 times greater than neutrophils, macrophages engulf lager and produce greater quantities of anti-foreign materials than neutrophils. Macrophages are more powerful phagocytes than neutrophils, each capable of destroying lesser than 100 bacteria. After the phagocytosis of antigenic foreign particulates, activated macrophages also act as antigen-presenting cells which migrate to the local lymph nodes to prime specific immunity. Resulting from that, the production of specific antibody is up-regulated, and in turn increases the opsonization and the highly efficient phagocytosis. To support neutrophils function, activated macrophages release TNF and IL-1 that act together to mediate neutrophil activation, lower pain threshold and blood pressure, and stimulate release of lipid mediators. IL-8 released from macrophages is an important chemotactic factor and stimulates the release of enzymes and ROS from neutrophils, leading to tissue damage and increased capillary permeability. In addition, monocytes-macrophages and another primed cell in lymph nodes secrete colony-stimulating factors (CSFs), which stimulate production of more white blood cells (WBCs) from bone marrow during inflammation. The increase of

WBCs helps to support and enhance the foreign-body clearance (Hansen, 1998; Peterson et al., 1995; Sommer, 2002).

Macrophages are the dominant cell in chronic inflammation. When acute inflammation progresses to the chronic phase, the long-life activated macrophages persist and can proliferate. Steady release of lymphocytes-derived chemokines and other cytokines is an important mechanism by which macrophages are recruited to or immobilized in chronic inflammatory sites. These mechanisms lead to fibroblast proliferation, with subsequent scar formation that in many cases replaces the normal connective tissue or functional parenchymal tissues of the involved structures. In the presence of IFN-γ which is released from lymphocytes, macrophages can fuse into large, multi-nucleated cells called giant cells. The accumulation of massing macrophages can induce the formation of granuloma, surrounded by lymphocytes where they wall off the indigestion of foreign material (Kumar et al., 2007; Sommer, 2002).

This section indicates that macrophages play an important role during inflammatory processes. They participate in both acute and chronic inflammation to initiate the inflammatory responses and to act as effective cells to destroy foreign particulates. The high accumulation of inflammatory macrophages and the high production of their releasing mediators at the inflammatory site contribute to promote cell toxicity and pathogenesis. Therefore, blocking of macrophage activation and their releasing factors is the aim of anti-inflammatory drugs.

1.2.3.2 Signaling Transduction of LPS-induced Toll-like Receptor 4

The toll-like receptor (TLR) family is the Pattern-Recognition Receptors (PRRs) that initiate inflammatory responses. The members of TLR family (Table 11) are characterized by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a Toll/IL-1 receptor (TIR) domain in their intracellular domain. TLR4 is critical for host defense against Gram-negative bacteria. LPS, a glycolipid component of the outer membrane of Gram-negative bacteria, exhibits the most potent immunostimulatory activity that leads to the production of numerous pro-inflammatory mediators such as TNF-α, IL-1, IL-6 and iNOS (Takeda et al., 2003; West et al., 2006).

Recognition of LPS requires other molecules in addition to TLR4. LPS binds to LPS-binding protein in serum and this LPS-LPS-binding protein complex is

subsequently recognized by CD14, a glycosylphosphatidylinositol-anchored molecule preferentially expressed in monocytes/macrophages and neutrophils. In addition, MD-2 is another molecule that associates with TLR4 to enhance the intracellular distribution of TLR4 and to act as a co-receptor for LPS binding. Upon recognition of LPS-LPS-binding protein complex together with MD-2 co-receptor, TLR4 initiates two major intracellular signaling pathways (Fig. 10), MyD88-dependent and TRIF-dependent pathways. The main role of the MyD88-dependent pathway downstream of TLR4 is to induce the expression of inflammatory cytokines, whereas the main TRIF-dependent pathway is to induce the expression of co-stimulatory molecules (Lee and Kim, 2007; Takeda et al., 2003).

MyD88-dependent pathway requires the recruitment of TIRAP and MyD88 throughout hemophilic TIR-TIR interaction. Then, MyD88 associates with two IL-1R-associated kinases (IRAK), IRAK4 and IRAK1, leading to the sequential activation of IRAK4, IRAK1 and TRAF6. After auto phosphorylation of MyD88/IRAK4/IRAK1 complex, IRAK1 and TRAF6 dissociate from the receptor complex and interact with the E2 ubiquitin-conjugating enzyme complex. The ubiquitination of dimerized TRAF6 leads to the recruitment and subsequent phosphorylation and activation of complex made up of TAK1 and two adaptor protein, TAB1 and TAB2. The activated TAK1 can phosphorylate two district signaling transductions, NF-κB activation pathway and MAPK pathway. In addition, TRAF6 also associates with ECIST which interacts with MEKK1 and activates NF-κB (Lee and Kim, 2007; West et al., 2006).

The TRIF-dependent pathway in TLR4 requires the recruitment of TRAM and TRIF. Then, TRIF initiates the signaling pathway, leading to the expression of inflammatory cytokines and Type I IFNs by interacting at least RIP1, TRAF6 and TRAF3. RIP1 interacts with the C-terminal of TRIF and this interaction is critical for TRAF6 recruitment and thus to TAK1 and NF-κB activation. In addition, the C-terminal of TRIF associates with TRAF6 which leads to the activation of TAK1 similarly found in MyD88-dependent pathway. TRAF3 can associate with TRIF and two noncanonical IKKs, IKKi (IKKε) and TBK1 and acts as a critical adaptor for TRIF-mediated IRF3 activation, leading to the production of IFN-β (West et al., 2006).

Both MyD88-dependent and TRIF-dependent pathways of TLR4 signaling regulate NF-κB activation and MAPK activation which participate in the expression of

pro-inflammatory mediators. In this study, the effects of purified compounds on pro-inflammatory mediator production are of interest. Therefore, the next sections consist of literature relating to signaling pathways that initiate the production of inflammatory mediators, including NF-κB activation and MAPK activation pathways.

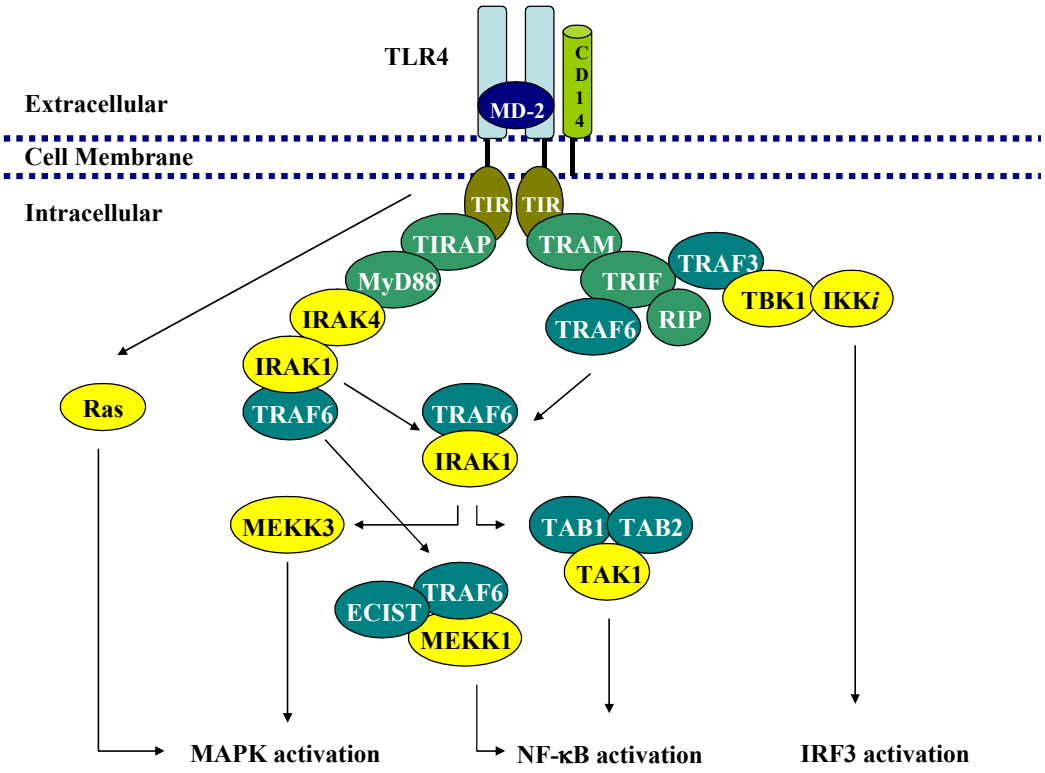

Figure 10. Toll-like receptor 4 signaling pathway (Takeda et al., 2003; West et al., 2006; Lee and Kim, 2007)

Table 11. Toll-like receptor family: their ligands and cell types (Takeda et al., 2003; Lee and Kim, 2007)

TLR Family	Cell Types	Ligands (Origin)
TLR1	Macrophage Conventional DC Neutrophil Mast cell	- Tri-acyl lipopeptide (bacteria, *Mycobacteria*) - Soluble factors (*Neisseria meningitides*)
TLR2	Macrophage Conventional DC Neutrophil Mast cell	- Lipoprotein/lipopeptide (a variety of pathogens) - Peptidoglycan (Gram-positive bacteria) - Lipoteichoic acid (Gram-positive bacteria) - Lipoarabinomanan (*Mycobacteria*) - A phenol-soluble modulin (*Sthaphylococcus epidermidis*) - Glycoinositolphospholipids (*Trypanosoma cruzi*) - Glycolipid (*Treponema maltophilum*) - Porins (*Neisseria*) - Zymosan (fungi) - Atypical LPS (*Leptospira interrogans*, *Porphyromonas gingivalis*) - HSP70 (host)
TLR3	Macrophage Conventional DC Endothelial cell Epithelial cell	- Double-strand RNA (virus) - Poly (I:C)

Table 11. Toll-like receptor family: their ligands and cell types (cont.)

TLR Family	Cell Types	Ligands (Origin)
TLR4	Macrophage	- LPS (Gram-negative bacteria)
	Conventional DC	- Taxol (plant)
	Neutrophil	- Fusion protein (RSV)
	Mast cell	- Envelope protein (MMTV)
	Eosinophil	- HSP60 (host)
		- HSP70 (host)
		- Type III repeat extra domain A of fibronectin (host)
		- Oligosaccharide of hyalurinic acid (host)
		- Polysaccharide fragments of heparin sulfate (host)
		- Fibrinogen (host)
TLR5	Monocytes	- Flagellin (bacteria)
	Conventional DC	
	Intestinal epithelial cell	
TLR6	Monocytes	- Di-acyl lipopeptides (*Mycoplasma*)
	Conventional DC	- Lipoteichoic acid (Gram-positive bacteria)
	Neutrophil	- Zymosan (yeast)
	Mast cell	
TLR7	Plasmatoid DC	- Immidazoquinoline (synthetic compounds)
	Neutrophil	- Loxoribine (synthetic compounds)
	Eosinophil	- Bropirimine (synthetic compounds)
		- Single-strand RNA (RNA virus)
		- Poly U RNA (synthetic compounds)

Table 11. Toll-like receptor family: their ligands and cell types (cont.)

TLR Family	Cell Types	Ligands (Origin)
TLR8	Monocyte Conventional DC Neutrophil Mast cell	Single-strand RNA (RNA virus) Resiquimod (synthetic compounds)
TLR9	Plasmatoid DC NK cell Neutrophil Eosinophil	- CpG DNA (bacteria, virus) - Hemozoin (*Plasmodium*)
TLR10	Plasmatoid DC B lymphocyte	- unknown ligand
TLR11	Macrophage Epithelial cell	- Profillin-like molecule (*Toxoplasma gondii*)

A. NF-κB Signaling Pathway

The Nuclear Factor-kappa B (NF-κB) family is a transcriptional factors that play an important role in a variety of biological functions, especially the immune system. In mammalian, the protein members of this family include RelA (p65), RelB, c-Rel, NF-κB1 (p50; p105) and NF-κB2 (p52; p100). These proteins have structurally conserved amino-terminal 300-amino-acid region which contains the dimerization, nuclear-localization and DNA-binding domain (Fig. 11). The NF-κB proteins show distinct roles in the regulation of development and immune function. Using knockout mice model (Table 12), Lack of RelA subunit results in embryonic death because of liver degeneration, whereas the lack of each of the other four subunits are associated with immunodeficiency. Not only that, NF-κB proteins control the expression of the immune-responsive gene, growth factors and transcription factors (Table 13) (Li and Verma, 2002; Siebenlist et al., 1994).

Normally, NF-κB protein is the dimer form, in which classic NF-κB (RelA/p50) is a well-known function and signaling activation. Classic NF-κB in the active form binds the sequences 5'-GGGRNNYYCC-3'. NF-κB proteins normally present in an inactive form by association with the proteins in the Inhibitor of NF-κB (IκB) family. IκB members contain multiple ankyrin repeats (Fig. 12) which are necessary for the binding to NLS of NF-κB. IκB-α plays the role of both sustaining NF-κB protein in an inactive cytoplasmic form, and ensuring the transient nature of the NF-κB response. As mentioned above, the ankyrin repeats of IκB-α interact with RHD and NLS of NF-κB resulting in NF-κB proteins being retained in cytoplasm. After activation, IκB-α is phosphorylated by serine-specific kinase (see below) at the N-terminal pair of serine residues and consequently degraded by proteasome. Therefore, NLS on NF-κB complex appear and induce the nuclear translocation of active NF-κB form. During active NF-κB complex binding to target sites, they also induce IκB-α expression. The newly synthesized IκB-α enters to nucleus using Nuclear Import Signal and interacts with nuclear NF-κB complex result in the inhibition of DNA-binding activity. After that, the nuclear IκB-α/NF-κB complexes are moved out from nucleus using Nuclear Export Signal (NES) and remain as an inactive NF-κB complex in cytoplasm (Baldwin, 1996; Whiteside and Israel, 1997).

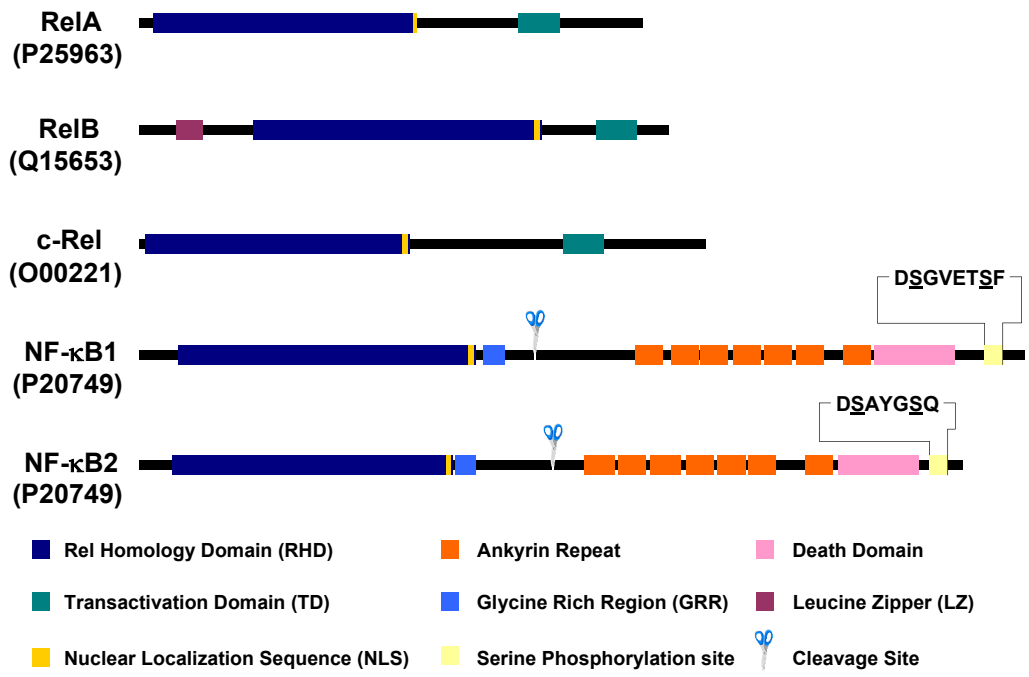

Figure 11. The schematic representation the member of NF-κB family of human protein

The NF-κB family comprises five members: RelA, RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). They have structurally conserved N-terminal Rel-homology domain (RHD) which contains the dimerization, nuclear localization sequence (NLS) and DNA-binding domain (Baldwin, 1996). RelA, RelB and c-Rel proteins have C-terminal non-homologous transactivation domain (TD). RelB has an additional leucine-zipper motif (LZ). In contrast, NF-κB1 and NF-κB2 are expressed in the precursor protein, p105 and p100 which are processed proteolytically to the mature p50 and p52 forms (Siebenlist et al., 1994). NF-κB1 and NF-κB2 contain the additional domains including glycine rich region (GRR), ankyrin repeat, death domain and serine phosphorylation sites (Li and Verma, 2002).

Table 12. Phenotype of knockout mice for NF-κB signaling components (Li and Verma, 2002)

Knockout gene	Phenotypes
NF-κB family	
RelA	- Die at E15.5-E16.5
	- TNF-dependent liver apoptosis
	- Defect in lymphocyte activation
RelB	- Die postnatally
	- Lack from multi-organ inflammation
	- Required for dendritic-cell development
c-Rel	- No development defects
	- Defects in lymphocyte and macrophage functions
NF-κB1	- Survival to adult
(both p105 and p50)	- Defect in lymphocyte activation
NF-κB2	- Survival to adult
(both p100 and p52)	- No mature B cells and defect in lymphocyte activation
	- Disruption of splenic and lymph-node architecture
RelA and NF-κB1	- Die at E13.5-E14.5
NF-κB1 and NF-κB2	- Die postnatally
	- Lack mature B cells and osteoblasts
NF-κB1 and RelB	- Die postnatally owing to immune deficiency
NF-κB1ΔC (p105)	- Die postnatally owing to immune deficiency
NF-κB2ΔC (p100)	- Die postnatally owing to immune deficiency

Table 12. Phenotype of knockout mice for NF-κB signaling components (cont.)

Knockout gene	Phenotypes
IκB family	
IκB-α	- Die postnatally owing to immune deficiency, Inflammatory dermatitis and granulocytosis - Constitutive NF-κB activity increase in lymphocytes but not in mouse embryonic fibroblast
IκB-α and NF-κB1	- Attenuated phenotype of IκB-α knockout mice
IκB-β knock-in to IκB-α knockout	- Rescues the defects of IκB-α□null mice
IκB-ε	- No defect in NF-κB activation - Lack severe immune defects
Bcl-3	- Disrupted splenic architecture
IKK complexes	
IKK1 (IKKα)	- Defects in keratinocyte differentiation, bone and limb development and mammary epithelial proliferation - No mature B cells - Impaired RANKL-induced NF-κB activation and NIK-induced p100 processing
IKK2 (IKKβ)	- Die at E13.5-E14.5 owing to TNF-dependent liver apoptosis - Impaired NF-κB activation by IL-1, TNF and LPS
IKK1 and IKK2	- Die at E11.5-12.5 owing to TNF-dependent liver apoptosis - No induced NF-κB activation in MEFs

Table 12. Phenotype of knockout mice for NF-κB signaling components (cont.)

Knockout gene	Phenotypes
IKK complexes	
NEMO (IKKγ)	- Die at E11.5-E12.5 owing to TNF-dependent liver apoptosis - No induced NF-κB activation in MEFs
Signaling components	
IKKi	- Impaired NF-κB activation by LPS, but not by TNF
TBK	- Die at E14.5 owing to TNF dependent liver apoptosis - Defective cytokine-induced expression of certain NF-κB target genes, but not IKK activation and IκB degradation
TRAF2	- Die postnatally - Mild effect on TNF-induced NF-κB activation
TRAF6	- Die posnatally - Osteoporosis and defective IL-1-, CD40-and LPS-mediated NF-κB activation
MEKK3	- Die at E10.5-E11.0 - Required for IKK and NF-κB activation by TNF and functioning downstream of RIP and TRAF2
NIK	- Survival to adult - Lack lymph nodes and Peyer's patches - Abnormal architecture of spleen and thymus - Required for LTβ-induced processing of p100 and NF-κB activation

Table 12. Phenotype of knockout mice for NF-κB signaling components (cont.)

Knockout gene	Phenotypes
Signaling components	
RIP	- Die postnatally owing to immune deficiency - No TNF-induced NF-κB activation
IRANK1	- Survival to adult - Defect in NF-κB activation by IL-1 and TIR signaling
GSK3β	- Die at E13.5-E14.5 with liver apoptosis - Defect in NF-κB activation by IL-1, but not IKK activation and IκB degradation
PKCζ	- Survival to adult - Defect in secondary lymphoid organ - Defect in NF-κB activation, but not IKK activation and IκB degradation in MEFs
PKCθ	- Survival to adult - Reduced proliferation of peripheral T cell - Impaired TCR-induced NF-κB activity in mature T cells

Table 13. NF-κB responsive genes (Siebenlist et al., 1994)

Cytokines	Interleukine-1 beta (IL-1β)
Chemokines	Tumor necrosis factor alpha (TNF-α)
Growth factors	Lymphotoxin (LT or TNF-β)
	Interleukin-8 (IL-8)
	IP-10
	Gro-α, -β and -γ
	MIP1-α
	MCP-1/JE
	RANTES
	Interleukine-2 (IL-2)
	Macrophage colony-stimulating factor (M-CSF)
	Granulocytes/macrophage colony-stimulating factor (GM-CSF)
	Granulocytes colony-stimulating factor (G-CSF)
	Erythropoietin
	Interferon-β and -γ
	Interleukine-6 (IL-6)
	Proenkephalin
	Transforming growth factor-β2 (TGF-β2)
Immunoreceptors	Immunoglobulin κ light chain (Ig-κ-LC)
	T cell receptor β chain
	Major histocompatibility complex class I (MHC-I)
	Major histocompatibility complex class II (MHC-II)
	β2-microglobulin
	MHC-II invariant chain
	Tissue factor-1
	Interleukin-2 receptor α chain
	CD7
Adhesion molecules	Endothelial-leukocyte adhesion molecule-1 (ELAM-1)
	Vascular cell adhesion molecule-1 (VCAM-1)
	Intracellular cell adhesion molecule-1 (ICAM-1)

Table 13. NF-κB-responsive genes (cont.)

Acute phase proteins	Angiotensinogen
	Serum amyloid A precursor
	Complement factor B
	Complement factor C4
	Urokinase-type plasminogen activator
Transcription factors and regulators	c-Rel
	NF-κB1 (p105)
	IκB-α
	Myc
	Interferon regulatory factor 1 (IRF-1)
	Interferon regulatory factor 2 (IRF-2)
	A-20
Viruses	Human immunodeficiency virus 1 (HIV-1)
	Human immunodeficiency virus 2 (HIV-2)
	Simian immunodeficiency virus (macaques) (SIV mac)
	Cytomegalovirus (CMV)
	Adenovirus
	Herpes simplex virus 1 (HSV-1)
	Human neutropic virus (JCV)
	Simian virus 40 (SV-40)
Others	Nitric Oxide Synthase (NOS)
	Apolipoprotein CIII
	Perforin
	Vimentin
	Decorin

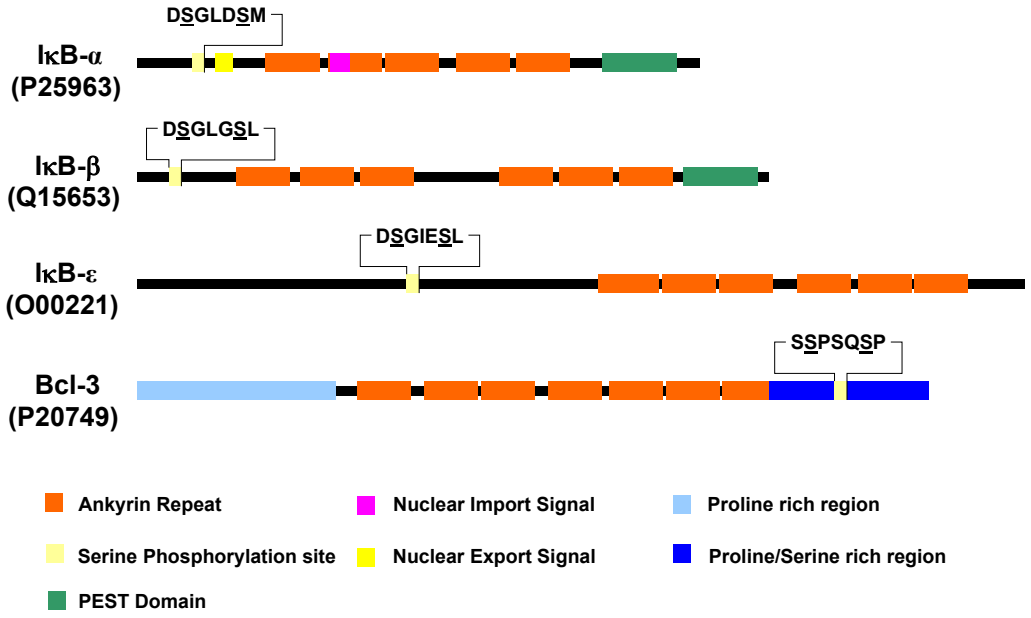

Figure 12. The schematic representation the member of IκB family of human protein

The inhibitor of NF-κB (IκB) family contains IκBα, IκBβ, IκBε and BCL-3. Proteins in this family are identified by the presence of many ankyrin repeats. The amino-acid sequences of the site of induced serine-phosphorylation are shown in this figure. The C-terminal of IκB contains PEST domain, the 42-amino acids rich in proline, glutamate, aspartate, serine and threonine residues that are the site of phosphorylation and has been implicated in regulating the stability of IκB as well as playing a role in the ability of IκB to inhibit DNA-binding by Rel/NF-κB complexes, perhaps via direct interaction with the DNA-binding domain (Whiteside and Israel, 1997; Li and Verma, 2002).

The initiation of NF-κB signaling pathway is started by the activation of IκB kinase (IKK) protein complex. IKK complex is composed of three polypeptides, IKK1 (IKKα), IKK2 (IKKβ) and NEMO (IKKγ) the structures of which are shown in Fig. 13. Activation of IKK complex depends on the phosphorylation of its IKK1 and IKK2 subunit (Karin, 1999). Linked with TLR4 signaling, The IKK1 and IKK2 subunits serve as a target for upstream activators involved in pro-inflammatory signaling and which are recruited to the complex via NEMO such as TAK1 (Takeda et al., 2003; West et al., 2006). The seminal event in the activation of NF-κB is the phosphorylation of IκB at specific N-terminal serine residues (Ser32 and Ser36 for IκBα) by activated IKK complex (Li and Verma, 2002). The phosphorylated IκBα leads to immediate recognition of IκBα F-box/WD40 $E3RS^{I_{\kappa B\alpha}}$/β-TRCP which consequently results in the polyubiquitinylation of IκBα at Lys21 and Lys22 by an SCF-type E3 (Karin and Ben-Neriah, 2000) as shown in Fig. 14. This modification targets rapid degradation by 26S proteasome, thereby releasing NF-κB dimmers from the NF-κB- IκB complex (Siebenlist et al., 1994). The degradation of its inhibitor exposes the NLS of NF-κB resulting in its binding to karyopherins and translocation of NF-κB to the nucleus. In the nucleus, active NF-κB binds to its DNA-binding motif and unregulates the transcriptional activity of target genes, especially IκBα. At the same time, the newly synthesized IκBα has an intrinsic NLS and can enter the nucleus and displace NF-κB from its DNA binding sites and transport NF-κB back to the cytoplasm using NES sequence which is located at the N-terminal of IκBα protein functions to expel the NF-κB-IκBα complex from the nucleus (Li and verma, 2002). The overall activation of NF-κB is shown in Fig. 15.

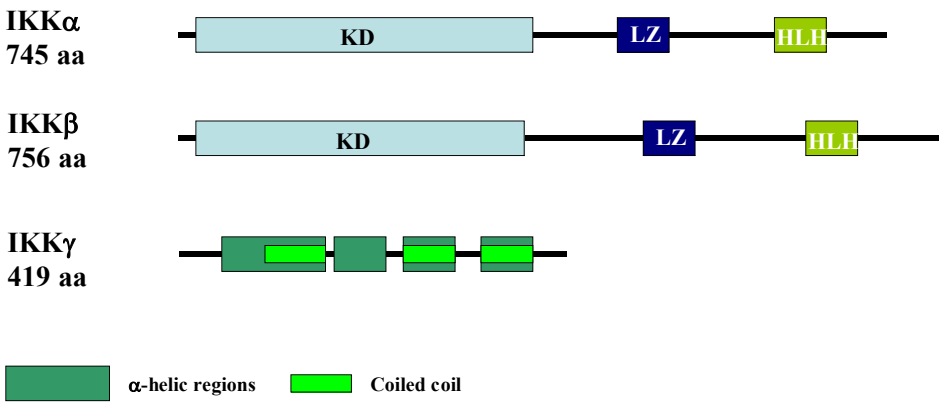

Figure 13. The three components of IKK

IKKα and IKKβ have very similar primary structures (52% overall identity) with protein kinase domain (KD) at their N-terminus, leucine zipper (LZ) and a helix-loop-helix (HLH) motif at their C-terminus. IKKγ (NEMO) does not contain a recognizable catalytic domain but is composed mostly of three large α-helical regions, including an LZ motif (Karin, 1999).

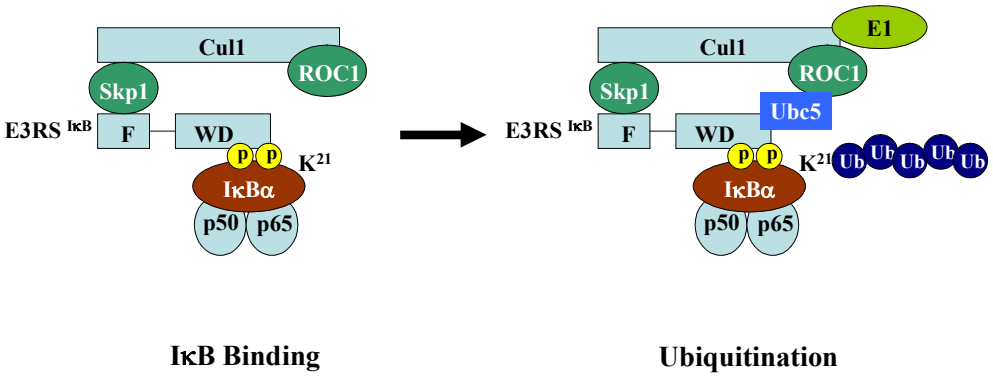

Figure 14. The IκB-E3 complex

The 700-kD degradation complex is composed of NF-κB, pIκB and other unknown proteins, which interact with a specific E3 complex. The E3 complex is composed of several proteins: Skp1, Cul1, Roc/Rbx1/Hrt1 and F-box protein, forming the SCF complex. The F-box $E3RS^{I\kappa B}$ protein is the variable component of SCF-complexes and function as the p-IκBα receptor component of E3 complex. Following pIκBα binding, the E3 complex associates with a specific E2, UBC5, and in the presence of E1 and ubiquitin catalyes the conjugation of ubiquitin to IκBα, as well as ubiquitin-ubiquitin conjugation. The latter function may be attributed to the Cul1:Roc1 complex (Karin and Ben-Neriah, 2000).

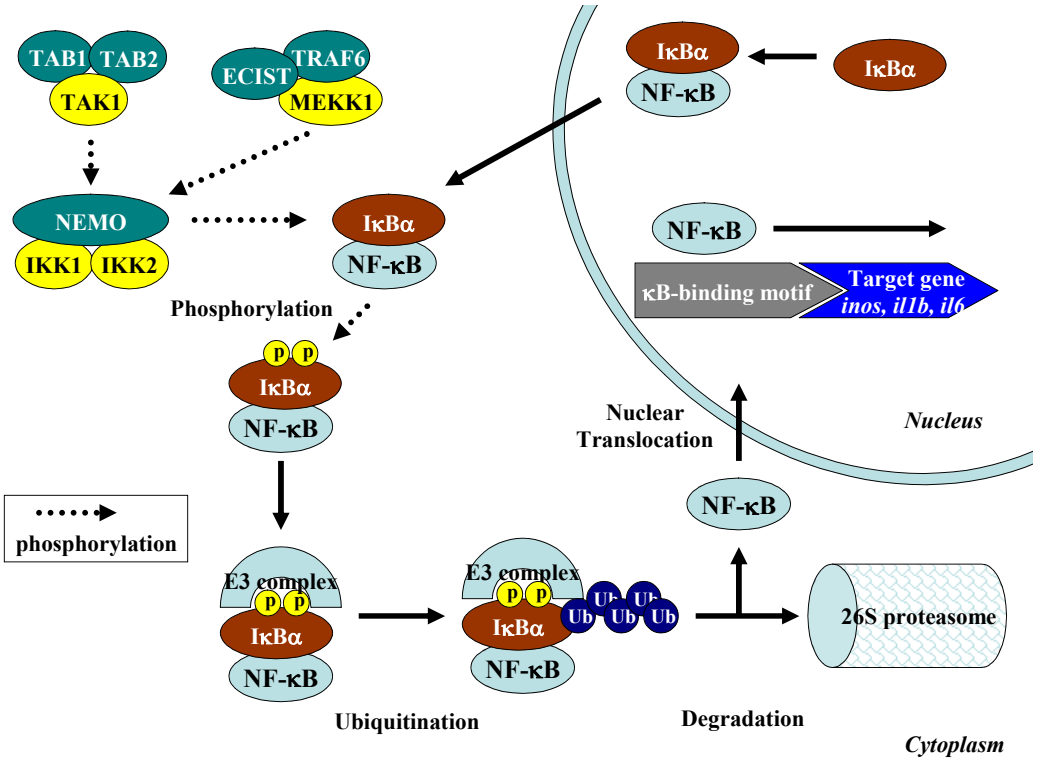

Figure 15. The overall activation processes of NF-κB signaling pathway

B. Mitogen-Activated Protein Kinase Signaling Pathway

Mitogen-activated protein kinases (MAPKs) are the highly conserved family of protein serine/threonine kinases, including ERK, JNK and p38 proteins. During LPS-induced TLR4, MAPKs are activated by two pathways (Guha and Mackman, 2001; West et al., 2006). JNK and p38 are activated by the downstream phosphorylation of MEKK3 which generates the activation of MKK4/7 and MKK3/6, respectively. LPS-induced TLR4 initiates the activation of Ras which consequently activates c-Raf, MEK1/2 and ERK1/2, respectively. However, the linkage between TAK1 and ERK activation is unclear. The activation of p38 and JNK is essential for the activation of transcription factors such as AP-1, CREB and Sp1 which participate in the transcription of inflammatory mediators (Hommes et al., 2003). The activation of p38 MAPK also enhances the assembly of p50/p65-TBP-CBP complex that binds to gene promoters and initiates the transcription (Guha and Mackman, 2001). The activation of ERK1/2 participates in the activation of IL-10 (Chanteux et al., 2007) and inhibits TBP binding activity during NF-κB activation (Xiao et al., 2002). The overall LPS-induced TLR4 through MAPK is shown in Fig. 16.

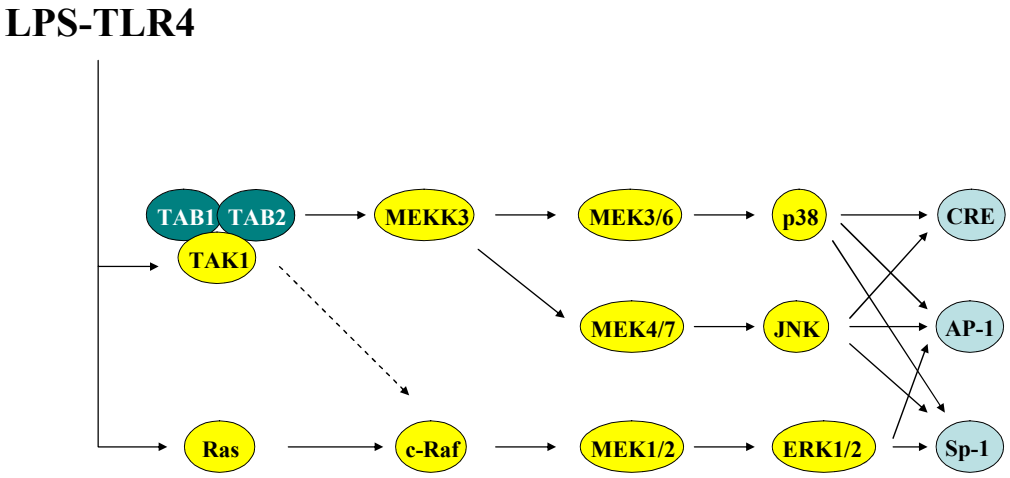

Figure 16. The overall MAPK activation processes during TLR4 signaling pathway (Guha and Mackman, 2001; Takeda et al., 2003)

1.2.4 *Dioscorea membranacea* (Pierre ex Prain & Burkill)

1.2.4.1 Description of *Dioscorea membranacea*

Dioscorea membranacea is a member of Dioscoreaceae. In Thailand, this plant is called Hua-Khao-Yen, Phak-Lum-Phua and Man-Moo. *D. membranacea* is distributed from Thailand westwards to north Burma, eastwards into Cambodia and southwards passing beyond the Isthmus of Kra into Malaysia. The rhizome has been used to treat cancer and inflammatory conditions for a long time in Thai Traditional Medicine. A description of *Dioscorea membranacea* is shown in Fig. 17. The rhizome is wide running, perhaps even up to 2 m. It is dark brown with white flesh. The stem is slightly ridged and unarmed. Leaves are deeply tirfid above a cordate base with a short acuminate 9 nerved, two primary nerves reaching the forerunner tip along with the midrib and the second pair reaching the tips of the lateral lobs. The petioles are 1/2-2/3 of length of the blade. Male flowers have small subsessile cymes with up to 4 flowers, sepals 1 mm long, and long-ovate. Stamens, alike the filaments insert just below the sepals 0.3 mm long. The anther is small and introrsely. Female flowers are downwardly directed spike-like racemes. Outer sepals are obovate, inner ones are lanceolate, and the inner are a little shorter than the outer. Style is short. Capsules are about 1-2 cm apart (Itharat, 2002; Saetung, 2006).

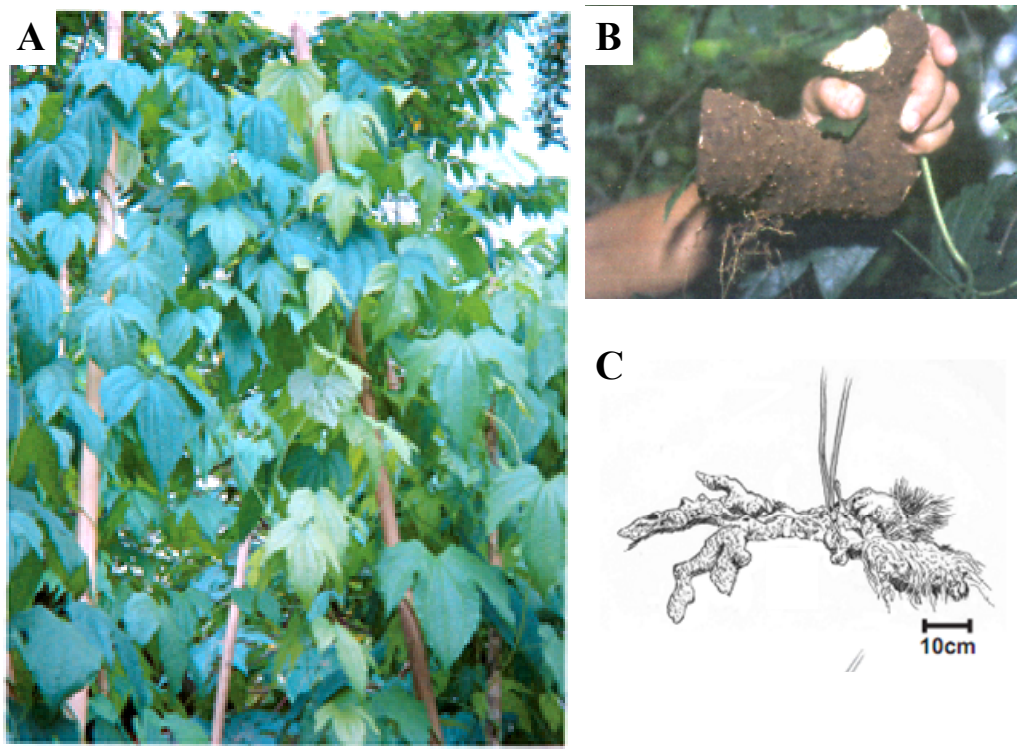

Figure 17. ***Dioscorea membranacea*** **(Pierre ex Prain & Burkill)**

(A) The aerial part of *Dioscorea membranacea* (Itharat, 2002) **(B)** The fresh rhizomes (Saetung, 2006) **(C)** The drawing picture of rhizomes (Maneenoon et al., 2008)

1.2.4.2 Biological Activities of *Dioscorea membranacea*

D. membranacea have shown biological activities including anti-oxidative activity, anti-inflammatory and anti-cancer. The ethanol extract of this plant exerted more potent biological activity than the aqueous extract as shown in Table 14. So, the ethanol extract was further investigated in terms of its chemical constituents, as showed in Figure 18. In addition, Dioscorealide A, Dioscorealide B and Dioscoreanone were the active compounds in the inhibition of NO, TNF-α and β-hexosamindase release, and the inhibition of HIV1 protease activity (Tewtrakul and Itharat, 2006; Tewtrakul et al., 2006; Tewtrakul and Itharat, 2007).

Table 14. The biological activities of *Dioscorea membranacea* (Pierre ex Prain & Burkill)

Biological Assay	IC_{50} (μg/ml)		Ref.
	Ethanol extract	Aqueous extract	
DPPH assay	16.52 ± 0.26	141.64 ± 1.61	(Itharat, 2002)
Lipid peroxidation assay	8.10 ± 1.21	742.17 ± 0.05	
Cytotoxicity			(Itharat et al., 2004)
• COR-L23	1.90 ± 0.10	10.30 ± 0.90	
• LS-174T	15.90 ± 1.10	3.30 ± 0.30	
• MCF-7	0.08 ± 0.20	0.04 ± 0.10	
β-hexosamindase	37.5	-	(Tewtrakul and Itharat, 2006)
Anti-HIV1 protease	48.0 ± 1.60	> 100	(Tewtrakul et al., 2006)
Nitric oxide release assay	23.6	57.8	(Tewtrakul and Itharat, 2007)

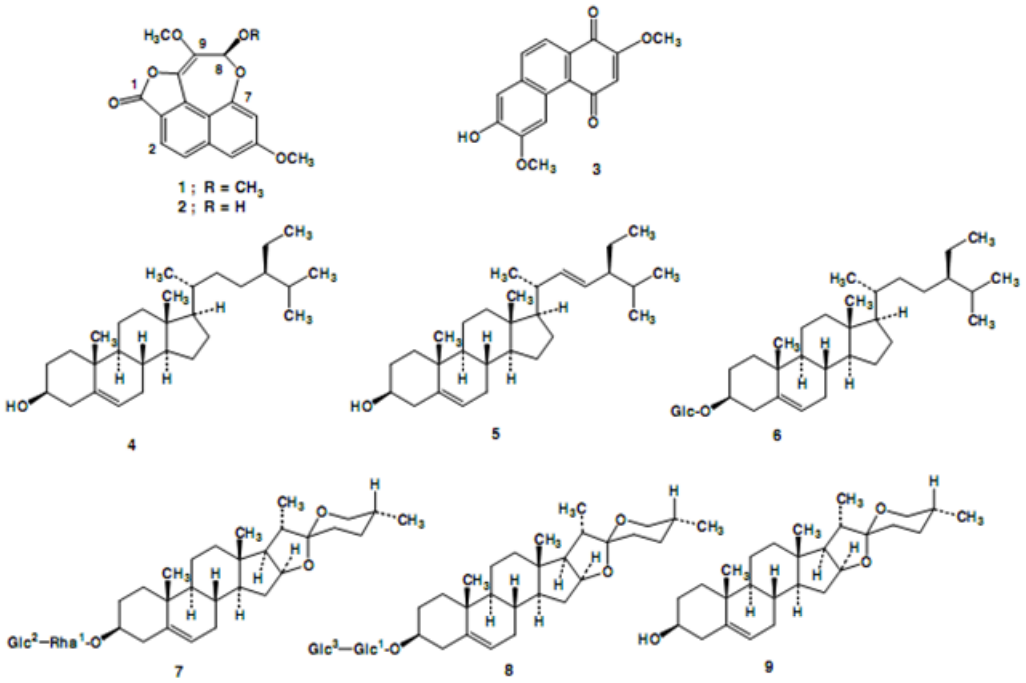

Figure 18. The chemical constituents of the ethanol extract from *Dioscorea membranacea* rhizome

The ethanol extract of *D. membranacea* contains Dioscorealide A **(1)**, Dioscorealide B **(2)**, Dioscoreanone **(3)**, β-Sitosterol **(4)**, Stigmasterol **(5)**, β-Sitosterol-3-*O*-β-D-glucopyranoside **(6)**, Diosgenin-3-*O*-α-L-rhamnosyl (1→2) -β-D-glucopyranoside **(7)**, Diosgenin-3-*O*-β-D-glucopyranosyl (1→3) -β-D-glucopyranoside **(8)** and Diosgenin **(9)** (Tewtrakul and Itharat, 2006)

1.2.4.3 Literature of *Dioscorea* Genus

Dioscorea is the largest genus that belongs to the family Dioscoreacea. It comprises 350-400 species which are present throughout tropical and subtropical regions, especially in West Africa, part of Central America and the Caribbean, the Pacific Islands and Southeast Asia (Caddick et al., 2002). The plants in genus *Dioscorea* is commonly known as Yam and has been a main food source. Some *Dioscorea* have been reported as a medicine (Table 15). The most common chemical compounds of genus *Dioscorea* are a steroidal saponin group with diosgenin as the aglycone. Table 16 shows the pure compounds isolated from *Dioscorea* genus.

Table 15. The part of uses and biological properties of genus *Dioscorea*

Species	Part of uses	Medicinal Property	Reference
D. alata	Tuber	- reduce hypertension by the inhibition of Angiotension Converting enzyme	(Hsu et al., 2002)
		- anti-oxidative effect in hyperhomocysteinemia rats	(Chang et al., 2004)
		- free-radical scavenging activity	(Chen et al., 2004)
		- induced cytokine expression in macrophage via TLR-4 activation	(Fu et al., 2006)
		- prevent liver and kidney damage by acetaminophen in rat	(Lee et al., 2002)
		- immunomodulating activity	(Liu et al., 2007b)
		- Estrogen receptor regulation	(Cheng et al., 2007)
D. batatas	Tuber	- anti-oxidative activity	(Hou et al., 2001)
		- induction of growth hormone release from rat pituitary cell	(Lee et al., 2007)
		- α-carbonic anhydrase dehydrogenase activity	(Hou et al., 1999)
		- tyrosin inhibitor activity	
		- allergen	(Hur et al., 2008)
		- immunostimulant	(Choi net al., 2004)
		- anti-inflammation	(Lee and Lim, 2008)

Table 15. The part of uses and biological properties of genus *Dioscorea* (cont.)

Species	Part of uses	Medicinal Property	Reference
D. bulbifera	Rhizome	- antitumor-promoting on mouse epidermal JB6 cell lines	(Gao et al., 2002)
	Tuber	- antitumor	(Tepommo et al., 2006)
		- leprosy treatment	
		- anti-typhoid activity	
	Fresh leave and stem	- reduced tumor mass of solid sarcoma 180 cells	(Komori, 1997)
		- to treat ulcer, hemorrhoid, dysentery and syphilis	(Narula et al., 2007)
D. collettii	Rhizome	- cervical carcinoma	(Hu et al., 1999)
		- carcinoma of urinary bladder	
		- renal tumor	
		- anti-mitotic agent for HepG2 liver cancer cells	(Wang et al., 2006)
		- cytotoxicity against 60 human cancer cell lines	(Hu and Yao, 2001)

Table 15. The part of uses and biological properties of genus *Dioscorea* (cont.)

Species	Part of uses	Medicinal Property	Reference
D. japonica		- prevent hyper cholesterolemia	(Chen et al., 2003)
D. nipponica	-	- asthma treatment	(Kwon et al., 2003)
		- Rheumatoid arthritis treatment	
		- bronchitis treatment	
D. opposita	Rhizome	- anorexia	(Satour et al., 2004b)
		- chronic diarrhoea	
		- diabetes	
		- seminal emission	
		- excessive leucorrhea	
		- improve insulin resistance in fructose-rich chaw-fed rats	(Hsu et al., 2007)
		- reduced blood glucose level in dexamethasone-induced insulin resistance	(Gao et al., 2007)
		- enhance insulin-induced glucose uptake in 3T3-L1 adipocytes by the regulation of GLUT4 expression	

Table 15. The part of uses and biological properties of genus *Dioscorea* (cont.)

Species	Part of uses	Medicinal Property	Reference
D. polygonoides	Tuber	- hypoglycemic effect	(McAnuff et al., 2005; McAnuff-Harding et al., 2006)
		- hypoglycemic activity	(Omoruyi, 2008)
		- hypolipidemic activity	
D. preussii	leave	- antileishmanial activity	(Lammidi et al., 2005)
D. pseudojaponica		- anticarcinogenic	(Lin et al., 2006)
		- antithrombotic	
		- antiviral	
		- hemolytic	
		- hypocholesterolemic	
		- hypoglycemic effects	
D. septemloba	Rhizome	- treatments of urethral and renal infections	(Liu et al., 2008)
		- rheumatism treatment	

Table 15. The part of uses and biological properties of genus *Dioscorea* (cont.)

Species	Part of uses	Medicinal Property	Reference
D. spongiosa	-	- stimulation of osteoblast proliferation - inhibition of osteoclast formation	(Yin et al., 2004)
D. tokoro	Root	- anti-inflammation - down regulation of inflammatory mediator in synoviocytes	(Kim et al., 2004)
D. villosa	Root	- induce VEGF-A expression and to promote angiogenesis in osteoblasts - up-regulates VEGF-A and promotes angiogenesis in preosteoblast-like cells by a hypoxia-inducible factor-1α-dependent mechanism involving the activation of Src kinase, p38 MAPK, and Akt signaling pathways via estrogen receptor.	(Yen et al., 2005)

Table 16. The chemical constituents of genus *Dioscorea*

Species	Part of plant	Chemical Constituents	Reference
D. alata	Tuber	- dioscorin (32 kDa protein)	(Hsu et al., 2002)
		- hydro-Q9 chromene	(Cheng et al., 2007)
		- γ-tocopherol-9	
		- RRR-α-tocopherol	
		- coenzyme Q9	
		- 1-feruloylglycerol	
D. batatas	Tuber	- dioscorin (32 kDa protein)	(Hou et al., 1999; Hou et al., 2001)
		- discorin (27 kDa proteins)	(Hur et al., 2008)
		- dioscin	(Lee et al., 2007)
D. bulbifera	Tuber	- Bafoudiosbulbins A and B	(Teponno et al., 2006)
		- tetracosanoic acid	
		- 1-(tetracosanoyl)-glycerol	
		- trans-tetracosanylferulate	
		- β-sitosterol	
		-3-*O*-β-D-glucopyranosyl-β-sitosterol	

Table 16. The chemical constituents of genus *Dioscorea* (cont.)

Species	Part of plant	Chemical Constituents	Reference
D. bulbifera	Rhizome	- kaempferol-3,5-dimethyl ether	(Gao et al., 2002)
		- caryatin	
		- (+)-catechin	
		- myricetin	
		- quercetin-3-*O*-galactopyranoside	
		- myricetin-3-*O*-galactopyranoside	
		- myricetin-3-*O*-glucopyranoside	
		- diosbulbin B	
	Fresh Leave and stem	- diosbulbin A and B	(Komori, 1997)
		- diosbulbin A 2-*O*-β-D-glucopyranoside	

Table 16. The chemical constituents of genus *Dioscorea* (cont.)

Species	Part of plant	Chemical Constituents	Reference
D. cayenensis	Rhizome	- 26-*O*-β-D-glucopyranosyl-3β,26-dihydroxy-20,22-seco-5(*R*)-furost-5-en-20,22-dione-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside	(Sautour et al., 2004a)
		- methyl protodioscin	
		- asperoside	
		- prosapogenin A of dioscin	
D. collettii	Rhizome	- hypoglaucin G	(Hu et al., 1999)
		- Pregna-5,16-dien-3β-ol-20-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside	
		- methyl protodioscin	(Wang et al., 2006)
		- methyl protogracillin	(Hu and Yao, 2001)

Table 16. The chemical constituents of genus *Dioscorea* (cont.)

Species	Part of plant	Chemical Constituents	Reference
D. nipponica	Root	- dioscin	(Kwon et al., 2003)
		- diosgenin	
		- dioscin	(Lin et al., 2007)
		- pseudoprotodioscin	
		- protodioscin	
		- methyl protodioscin	
	Rhizome	- dioscin	(Liu et al, 2007a)
		- prodioscin	
		- gracillin	
D. opposita	Rhizome	- 3,4,6-trihydroxyphenanthrene-3-*O*-β-D-glucopyranoside	(Satour et al., 2004b)
		- soyacerebroside I	
		- adenosine	
		- β-sitosterol	
		- palmitic acid	
		- palmitoyloleoylphosphatidylcholine	

Table 16. The chemical constituents of genus *Dioscorea* (cont.)

Species	Part of plant	Chemical Constituents	Reference
D. panthaica		- spirostanol saponins - furostanol saponins	(Li et al., 2006)
D. polygonoides	Tuber	- Δ^3 diosgenin - diosgenin - pennogenin - stigmasterol - β-sitosterol	(McAnuff et al., 2005)
D. pseudojaponica		- methyl protodioscin - methyl protogracillin - dioscin - gracillin	(Yang et al., 2003)

Table 16. The chemical constituents of genus *Dioscorea* (cont.)

Species	Part of plant	Chemical Constituents	Reference
D. pseudojaponica		- 26-*O*-β-D-glucopyranosyl-22 α -methoxyl-(25*R*)-furost-5-en-3β,26-diol 3-*O*- α -L-rhamnopyranosyl-(1→2)-*O*-[[α -L-rhamnopyranosyl-(1→4)]-*O*-[α -L-rhamnopyranosyl-(1→4)]]-β-D-glucopyranoside - methyl protodioscin - methyl protogracillin - (25*R*)-spirost-5-en-3β-ol 3-*O*- α -L-rhamnopyranosyl-(1→2)-*O*-[[α -L-rhamnopyranosy-(1→4)]-*O*-[α -L-rhamnopyranosyl-(1→4)]]- β-D-glucopyranoside - dioscin - gracillin	(Lin et al., 2006)
D. septemloba	Rhizome	- dioseptemlosides A-H	(Liu et al., 2008)
D. villosa	Root	- diosgenin	(Yen et al., 2005)

1.3 Objective

This study aimed to evaluate the mechanisms of Dioscorealide B and Dioscoreanone on anti-inflammatory activity in LPS-induced RAW 264.7 macrophages, including:

1. The effect of Dioscorealide B and Dioscoreanone on nitric oxide production
2. The effect of Dioscorealide B and Dioscoreanone on the mRNA expression of iNOS, IL-1β, IL-6 and IL-10
3. The effect of Dioscorealide B and Dioscoreanone on NF-κB activation
4. The effect of Dioscorealide B and Dioscoreanone on ERK1/2 MAPK activation

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

2.1.1 Dioscorealide B and Dioscoreanone

Dioscorealide B and Dioscoreanone (Appendix A-1) isolated from the ethanol extract of *Dioscorea membranacea* rhizomes were obtained from Assoc. Prof. Dr. Arunporn Itharat. The structures of these two compounds were elucidated by comparing ^{1}H- and ^{13}C-NMR spectral data which had been reported (Itharat et al., 2003). The stock solution preparations of crude extracts are shown in Appendix B-1.

2.1.2 Cell line and cell culture

RAW 264.7 mouse macrophages (TIB-71) were purchased from American Type Culture Collection (ATCC). The cell line description is shown in Appendix A-2. These cells were grown at 37 °C with 5% CO_2 using complete Dulbecco's Modified Eagle Medium (DMEM, Appendix B-2) which was purchased from GIBCO (Grand Island, NY, USA). The DMEM was supplemented with 10% fetal bovine serum purchased from BIOCHROM AG (Berlin, Germany) and 100 unit/ml of penicillin-streptomycin purchased from GIBCO (Grand Island, NY, USA).

2.1.3 Stimulator and inhibitors

Lipopolysaccharide (LPS) from *Escherichia coli* serotype O111: B4 was used to stimulate the production of nitric oxide and the expression of iNOS, IL-1β, IL-6 and IL-10. *N*-acetyl cysteine (NAC) was used to inhibit arylating reaction of quinone compounds. Dicoumarol (Dic) was used to inhibit the activity of NAD(P)H: Quinone oxidoreductase (NQOR). These three compounds were purchased from SIGMA (St. Louis, MO) and the preparations are shown in Appendix B-3.

2.1.4 Chemicals and reagents

2.1.4.1 Griess's assay reagents

N-(1-napthyl)-ethylenediamine dihydrochloride, Sulfanilamide and o-phosphoric acid (H_2PO_4) were used to prepare Griess's reagent (Appendix B-4) to determine nitrite concentration. Sodium nitrite ($NaNO_2$) was used as a standard. All of these compounds were purchased from MERCK (NJ, USA).

2.1.4.2 MTT assay reagent

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was used to determine the mitochondrial respiration (Appendix B-5). This chemical was purchased from SIGMA (St. Louis, MO).

2.1.4.3. RT-PCR reagents

TRIZOL reagent, M-MLV reverse Transcriptase, RNasOUT, *Taq* DNA polymerase and primers (Table 17) were used in semi quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to determine the mRNA expression of iNOS, IL-1β, IL-6, IL-10 and β-actin (Appendix B-6). These reagents were purchased from Invitrogen (Carlsbad, CA). Oligo dT_{16} used to reverse transcription was purchased from Amersham Biosciences (Piscataway, NJ).

2.1.4.4 Reporter assay reagents

pNFκB-Luc Reporter plasmid and luciferase assay kit were purchased from Stratagene (La Jolla, CA). FuGene6 Transfection reagent was purchased from Roche Applied Science (Mannheim, Germany).

2.1.4.5 Western blot reagents

RIPA lysis buffer, Protease Cocktail Inhibitors and antibodies including anti-IκBα, anti-p-IκBα, anti-ERK1, anti-ERK2 and anti-p-ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin was purchased from SIGMA (St. Louis, MO). The Bradford protein assay kit was purchased from Bio-RAD (Hercules, CA).

Table 17. Primer sequences for amplification

Targets		Primer sequence	Product (bp)	Tm (℃)
β-Actin	F-primer	5'-GACTACCTCATGAAGATCCT-3'	510	45
	R-primer	5'-CCA CAT CTG CTG GAAGGTGG-3'		
iNOS	F-primer	5'-ATGTCCGAAGCAAACATCAC-3'	450	45
	R-primer	5'-TAATGTCCAGGAAGTAGGTG-3'		
IL-1β	F-primer	5'-AAGCTCTCCACCTCAATGGA-3'	301	45
	R-primer	5'-TGCTTGTGAGGTGCTGATGT-3'		
IL-6	F-primer	5'-TTCCATCCAGTTGCCTTCTTGG-3'	360	45
	R-primer	5'-CTTCATGTACTCCAGGTAG-3'		
IL-10	F-primer	5'-GTGAAGACTTTCTTTCAAACAAAG-3'	273	45
	R-primer	5'-CTGCTCCACTGCCTTGCTCTTATT-3'		

2.2 Methods

2.2.1 Inhibitory effect of Dioscorealide B and Dioscoreanone on LPS-induced nitric oxide production in RAW 264.7 cells using Griess's reagent

RAW 264.7 cells (1×10^5 cells/well) were seeded in a 96-well flat bottom plate for 24 hrs. The culture medium was removed and replaced with fresh medium containing Dioscorealide B and Dioscoreanone at various concentrations. After 30 min, RAW 264.7 macrophages were challenged with 2.5 μg/ml of LPS and incubated for 24 hrs. The nitrite concentration was measured in the culture supernatant using Greiss's assay (Campa et al., 2005). Briefly, a hundred micro liters of each supernatant was incubated with 50 μl of sulfanilamide solution for 15 min at room temperature. Then, 50 μl of NED solution was added and left at room temperature for 15 min. The absorbance at 570 nm was determined by a microplate reader. The quantification of nitrite was standardized with 0-100 μM $NaNO_2$. The 50% Inhibitory Concentration of the test sample (IC_{50}) was obtained by linear regression analysis of dose-response curve plotting between the percentage of nitric oxide inhibition and the sample concentration of three independent assays (Appendix C-1).

2.2.2 Measurement of Cell function and Viability using MTT Assay

RAW 264.6 cells that were exposed to Dioscorealide B and Dioscoreanone were used to determine the mitochondrial function and viability using MTT assay (Appendix C-2). Briefly, RAW 264.7 cells were washed twice with PBS and incubated with 100 μl of MTT working solution at 37 ℃ for 1 hrs. Under light protection, the dark blue crystals of formazan (MTT metabolites) were dissolved with 200 μl of dimethyl sulfoxide (DMSO) at room temperature for 30 min and read at 570 and 630 nm using a microplate reader (Yun et al., 2003). The viable cells were expressed as a percentage of survival.

2.2.3 Screening the effect of Dioscorealide B and Dioscoreanone on signaling transduction

Raw 264.7 cells (1×10^5 cells/well) were seeded in a 96-well flat bottom plate for 24 hrs. The culture medium was removed and replaced with fresh medium. Dioscorealide B (3.0 μM) and Dioscoreanone (2.5 μM) were added every 3 hrs before and after challenging with 2.5 μg/ml LPS. After 24-hrs of LPS stimulation, cell culture mediums were analyzed for nitrite concentration (section 2.2.1). The results were compared to the stimulation with LPS alone and time zero of LPS stimulation in the presence of either 3.0 μM Dioscorealide B or and 2.5 μM Dioscoreanone.

2.2.4 The effect of Dioscorealide B and Dioscoreanone on inflammatory mediator mRNA expression using RT-PCR

RAW 264.7 cells (2×10^6 cells/well) were seeded in a 6-well plate for 24 hrs. The medium was removed and replaced with fresh medium containing various concentrations of Dioscorealide B and Dioscoreanone for 30 min. Then, cells were stimulated with 2.5 μg/ml of LPS. After 9 hrs of LPS exposure, cells were harvested and washed twice with cold PBS. Total RNA was isolated with a TRIZOL reagent according to the supplier's instructions (Appendix C-3). Total RNA (2.0 μg) from each sample were reverse transcribed with M-MLV reverse transcriptase at 37 °C for 50 min and finalized at 72 °C for 15 min (Appendix C-4). The cDNAs were amplified with *Taq* polymerase using the appropriate oligonucleotide primers complementary to iNOS, IL-1β, IL-6, IL-10 and β-Actin (Appendix C-5). The PCR was carried out in a thermal cycler (BIO-RAD, Hercules, CA) under the following conditions: initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 45 sec, 45 °C for 30 sec, 72 °C for 60 sec, and final extension at 72 °C for 10 min. PCR products were fractionated on 1.8% agarose in 0.5× TBE gels containing 2.5 μg/ml ethidium bromide (Appendix C-6). The gel image was captured and the intensity of the PCR products was analyzed by an automated gel doc system (Bioimagine System, Syngene, USA). Band intensity was measured using SiconImage program.

2.2.5 NF-κB binding activity using NF-κB-driven luciferase Reporter Assay

RAW 264.7 cells (1×10^5 cells/well) were seeded in a 48-well plate and allowed to adhere for 24 hrs. Cells (40-50% confluent) were transfected with 0.2 μg of pNFκB-Luc plasmid using 0.3 μl of Fugene6 transfection reagent for each well and then incubated for 24 hrs (Appendix C-7). Then, the cell culture medium was removed and replaced with fresh medium containing various concentrations of Dioscorealide B or Dioscoreanone for 30 min, followed by co-incubation with 2.5 μg/ml of LPS for 6 hrs. RAW 264.7 cells were extracted and the luciferase activity was measured using luciferase assay kits following the manufacturer's protocol (Appendix C-8). The chemiluminescence light intensity was detected using Tropix TR 717 Luminometer Plate Reader (Applied Biosystems, Foster City, CA).

2.2.6 IκBα and ERK1/2 protein activation using Western blot analysis

RAW 264.7 cells (1×10^7 cells) were cultured in a 10 cm-dish and allowed to adhere for 24 hrs. After treatment, the cells were washed twice with cold PBS and lysed in RIPA lysis (Appendix C-9). Cell lysates were rocked for 1 hr at 4 °C followed by a 15 min centrifugation at 10,000 ×g. The total protein concentration was determined by the Bradford method and standardized with BSA (Appendix C-10). Protein samples (100 μg) were separated on 12% SDS-polyacrylamide gels electrophoresis and transferred to a nitrocellulose membrane. The membranes were subsequently immuno-blotted with either anti-p-IκBα, anti-IκBα, anti-p-ERK1/2, anti-ERK1/2 or anti-Actin (Appendix C-11). The immuno-reactive protein bands were visualized on CL-XPosure film using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

2.2.7 Statistical Analysis

All assays were carried out in triplicate with a minimum of three independent experiments and results were expressed as the mean ± S.E.M. Comparison data were analyzed by ANOVA. The p value < 0.05 is considered significant.

CHAPTER 3

RESULTS

3.1 The effect of Dioscorealide B and Dioscoreanone on LPS-induced nitric oxide production in RAW 264.7 cells

Nitric oxide is one of the toxic agents that release during inflammation from macrophages. In this study, LPS-induced RAW 264.7 murine macrophage was used as the *in vitro* model to determine the effect of Dioscorealide B and Dioscoreanone (Figure 19) on the production of nitric oxide. The exposure of LPS to RAW 264.7 macrophage cells induces the production of nitric oxide that is converted to nitrite, corresponding to nitric oxide concentration. The accumulation of nitrite in cell culture medium is commonly determined using Griess's assay.

As shown in Figure 20A, the spontaneous levels of nitrite were 0.14 ± 0.09 and 0.30 ± 0.21 μM in the absence and presence of 0.2% DMSO, respectively. Upon 2.5 μg/ml of LPS stimulation, nitrite concentration rose significantly to 9.27 ± 1.69 μM and 7.57 ± 1.30 μM in the absence and presence of 0.2% DMSO, respectively. The 30-minute pre-incubation of RAW 264.7 cells with Dioscorealide B, prior to activation with 2.5 μg/ml of LPS, significantly suppressed the production of nitrite level in a concentration-dependent manner. When compared to LPS activation *per se*, Dioscorealide B exhibited the IC_{50} values at 2.85 ± 0.62 μM. The suppression was not due to chemically induced cytotoxicity at any dosage below 16 μM (Figure 20B).

In Figure 21A, RAW 264.7 cells in a resting state spontaneously released 0.70 ± 0.28 μM and 0.85 ± 0.27 μM of nitrite into the culture media in the absence and presence of 0.2% DMSO, respectively. Upon exposure to LPS alone or in the presence of DMSO, the nitrite levels increased markedly to 12.53 ± 1.48 μM and 10.29 ± 0.32 μM, respectively. A half hour of pretreatment with Dioscoreanone inhibited LPS-induced nitrite level in a concentration-dependent manner. The IC_{50} values of Dioscoreanone were 2.50 ± 0.64 μM. Dioscoreanone, at the tested concentration, did not show cytotoxicity to RAW 264.7 cells, indicating that the inhibitory effects of Dioscoreanone were not due to non-specific cell cytotoxicity (Figure 21B)

These results showed that both Dioscorealide B and Dioscoreanone inhibited LPS-induced nitrite accumulation. Therefore, Dioscorealide B and Dioscoreanone act as anti-nitric oxide production agents. Then, these two compounds were subjected to screen the inhibition of nitric oxide production at the signal transduction. The time-course suppressive effect of Dioscorealide B and Dioscoreanone on LPS-induced nitric oxide production is demonstrated in Figure 22. Dioscorealide B at 3.0 μM and Dioscoreanone at 2.5 μM were added to the RAW 264.7 cells at various time points, from 3 hrs pre-treatment to 18 hrs after stimulation with LPS. Both Dioscorealide B and Dioscoreanone significantly reduced nitric oxide inhibition when each compound was added for more than 3 hrs after LPS stimulation (## $p < 0.01$). In addition, the significant inhibitory effect of Dioscorealide B was found at all time points up to 15 hrs (Figure 22A), whereas the significant suppressive effect of Dioscoreanone was observed at 0 to 9 hrs (Figure 22B).

The experiments in this section suggest that Dioscorealide B and Dioscoreanone may inhibit LPS-induced nitric oxide production through the suppression at the signal transductions. This hypothesis can be proved by determining the expression of iNOS.

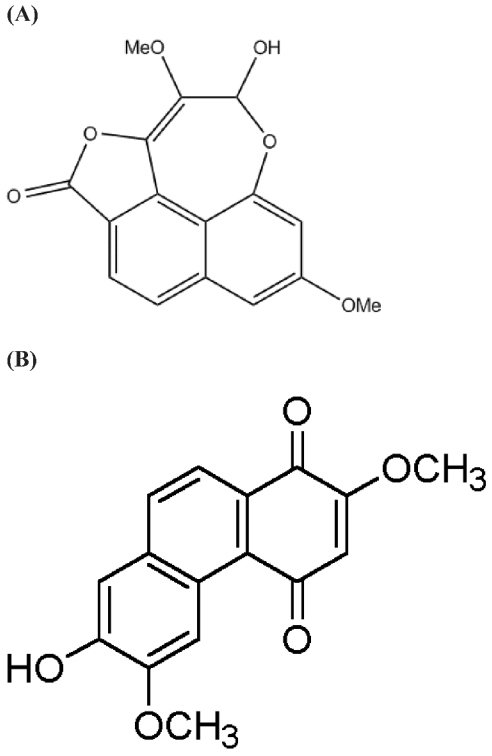

Figure 19. Structure of Dioscorealide B (A) and Dioscoreanone (B) isolated from the rhizomes of *Dioscorea membranacea* (Pierre ex Prain & Burkill)

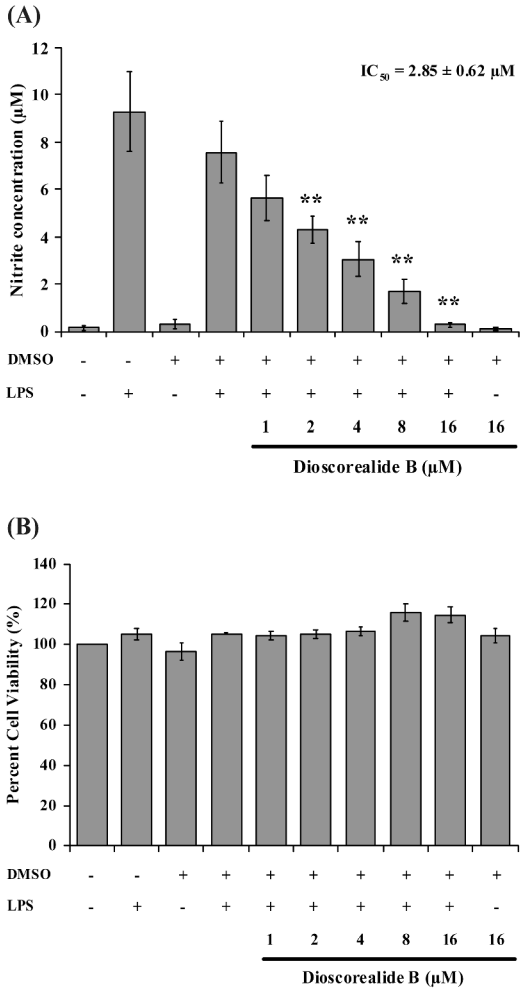

Figure 20. The effects of Dioscorealide B on the LPS-induced nitric oxide production in RAW 264.7 macrophages

(A) RAW 264.7 cells (1×10^5 cells/well) were seeded in a 96-well plate and allowed to adhere for 24 hrs. The adhered cells were pretreated with various concentrations of Dioscorealide B for 30 min prior to stimulation with 2.5 μg/ml LPS for 24 hrs. Nitrite in the culture medium was measured using Griess's assay. **(B)** The viability of the remaining cells after the removal of supernatants was determined by MTT assay. The accumulated nitrite production was measured at 24 hrs after LPS stimulation. The values were expressed as a mean ± S.E.M. from four independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with LPS and DMSO exposure (the 4^{th} bar).

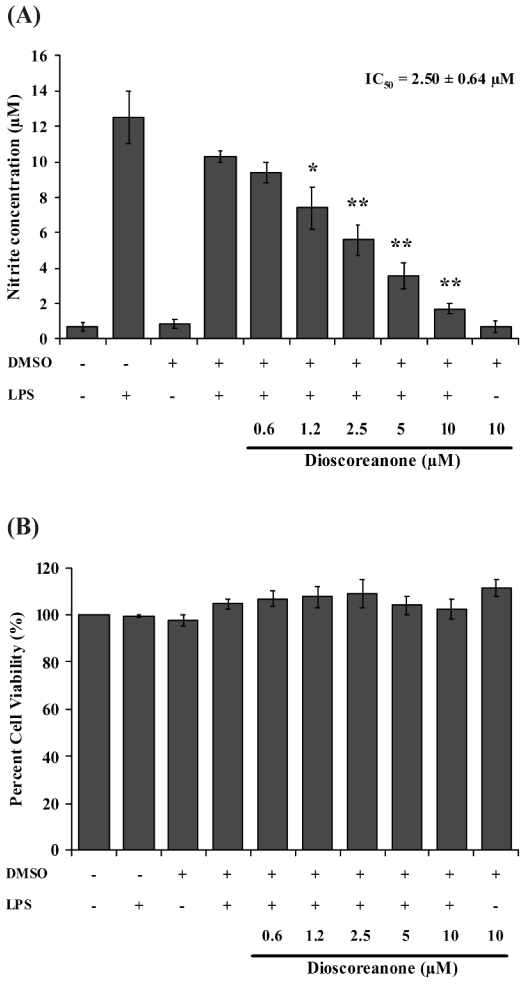

Figure 21. The effects of Dioscoreanone on the LPS-induced nitric oxide production in RAW 264.7 macrophages

(A) RAW 264.7 cells (1×10^5 cells/well) were seeded in a 96-well plate and allowed to adhere for 24 hrs. The adherent cells were pretreated with various concentrations of Dioscoreanone for 30 min prior to stimulation with 2.5 μg/ml LPS for 24 hrs. Nitrite in the culture medium was measured using Griess's assay. **(B)** The viability of the remaining cells after the removal of supernatants was determined by MTT assay. The accumulated nitrite production was measured at 24 hrs after LPS stimulation. The values were expressed as a mean ± S.E.M. from four independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with LPS and DMSO exposure (the 4^{th} bar).

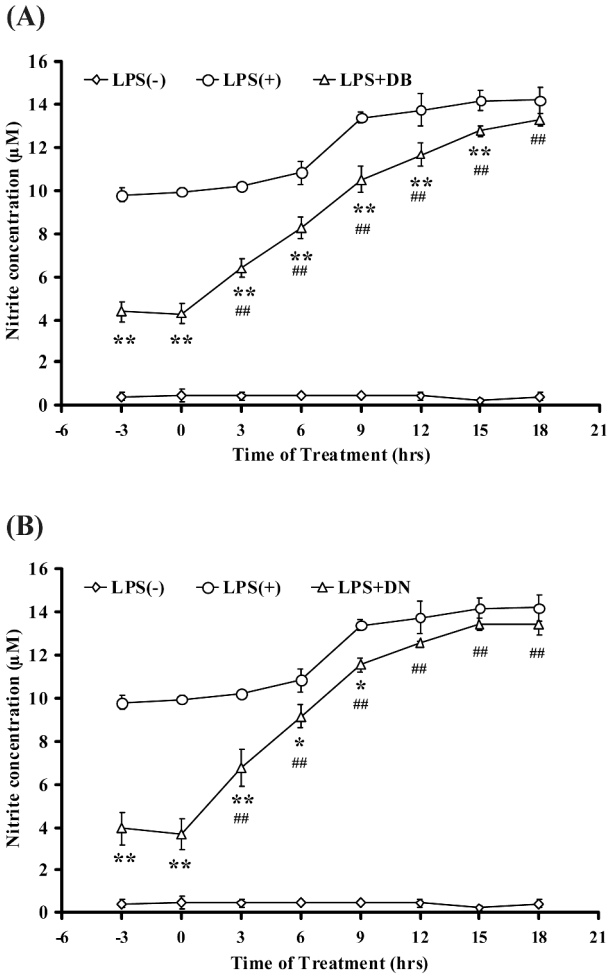

Figure 22. The time-course effect of Dioscorealide B and Dioscoreanone on the LPS-induced nitric oxide production in RAW 264.7 macrophages

RAW 264.7 cells (1×10^5 cells/well) were seeded in a 96-well plate and allowed to adhere for 24 hrs. **(A)** 3.0 μM Dioscorealide B (DB) and **(B)** 2.5 μM Dioscoreanone (DN) were added at sequential time-points every 3 hrs before and after LPS (2.5 μg/ml) activation. Accumulated nitrite production was measured at 24 hrs after LPS stimulation. The values were expressed as a mean ± S.E.M. from four independent experiments performed in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared with LPS and DMSO exposure (LPS-). ## $p < 0.01$ compared with LPS and sample exposure at time zero(LPS+DN at T_0).

3.2 The effect of Dioscorealide B and Dioscoreanone on the mRNA expression of iNOS and inflammatory cytokines in LPS-induced RAW 264.7 cells

In Section 3.1, the production of nitric oxide was inhibited by the treatment of Dioscorealide B and Dioscoreanone. Normally, LPS inducing the production of nitric oxide needs the signaling protein to initiate the expression of iNOS enzyme. When LPS-stimulated RAW264.7 macrophages were exposed to Dioscorealide B and Dioscoreanone at 3-hour after LPS-stimulation, the inhibition by these compounds decreased. It can be hypothesized that Dioscorealide B and Dioscoreanone suppress nitric oxide production at the signaling transduction of LPS-induce RAW 264.7 cells. Normally, the production of nitric oxide depends on the expression of iNOS. In addition, the other cytokines including IL-1β, IL-6 and IL-10, are induced in the response of LPS exposure. Therefore, the mRNA expression of iNOS, IL-1β, IL-6 and IL-10 was evaluated by RT-PCR.

As shown in Figures 23 and 24, iNOS mRNA transcription was slightly detectable in resting RAW 264.7 cells. It increased markedly by at least 1.5-fold from the basal level upon exposure to LPS. In Figure 23, 6.0 μM Dioscorealide B significantly suppressed iNOS transcription with 75.23 ± 7.02% inhibition. In Figure 24, 5.0 μM Dioscoreanone significantly attenuated LPS-induced expression of iNOS transcription with 89.21 ± 15.64% (Figure 24) inhibition. In addition, 6.0 μM Dioscorealide B suppressed the transcription of IL-1β, IL-6 and IL-10 in a concentration-dependent manner as shown in Figure 23. The percentage of inhibition for IL-1β, IL-6 and IL-10 were 71.18 ± 2.54, 97.08 ± 3.01 and 71.75 ± 4.09, respectively. In contrast, the inhibitory effect of Dioscoreanone on IL-10 mRNA expression was not found. Dioscoreanone significantly inhibited the expression of IL-1β and IL-6 with 43.00 ± 0.82 and 85.08 ± 2.60, respectively (Figure 24).

The results in this section showed that Dioscorealide B and Dioscoreanone inhibited the expression of iNOS mRNA. This result supported the hypothesis that Dioscorealide B and Dioscoreanone inhibit nitric oxide production through the suppression of iNOS. In addition, the mRNA expression of IL-1β and IL-6 was reduced by Dioscorealide B and Dioscoreanone. Only Dioscorealide B could inhibit IL-10 mRNA expression.

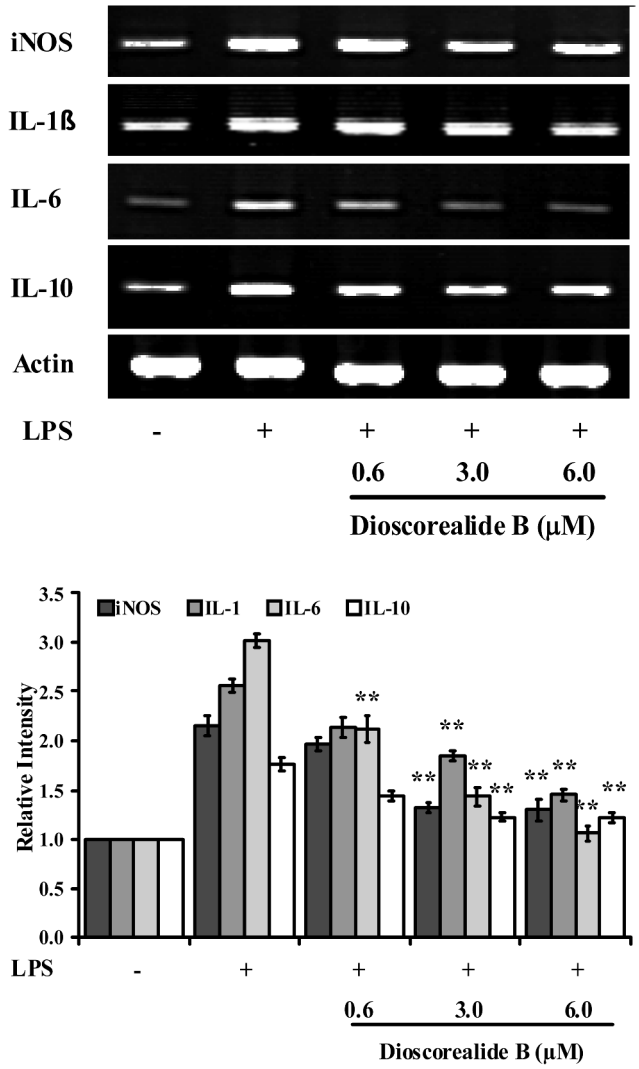

Figure 23. The effect of Dioscorealide B on LPS-induced mRNA expression of iNOS and inflammatory cytokines in RAW 264.7 macrophages

RAW 264.7 cells (2×10^6 cells/well) were seeded in 6-well plate and allowed to adhere for 24 hrs. The adhered cells were pretreated with various concentrations of Dioscorealide B for 30 min prior to stimulation with 2.5 μg/ml LPS for 9 hrs. The total RNA was isolated and amplified by semi quantitative RT-PCR. The band intensity was expressed as a mean ± S.E.M derived from three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with LPS stimulation (the 2nd lane).

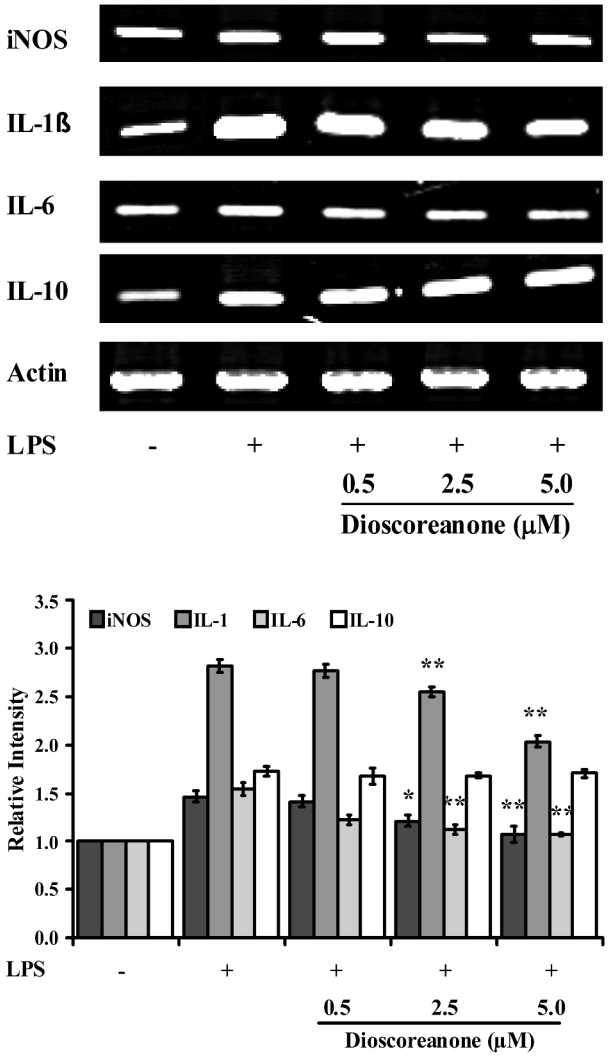

Figure 24. The effect of Dioscoreanone on LPS-induced mRNA expression of iNOS and inflammatory cytokines in RAW 264.7 macrophages

RAW 264.7 cells (2×10^6 cells/well) were seeded in 6-well plate and allowed to adhere for 24 hrs. The adhered cells were pretreated with various concentrations of Dioscoreanone for 30 min prior to stimulation with 2.5 μg/ml LPS for 9 hrs. The total RNA was isolated and amplified by RT-PCR. The band intensity was expressed as a mean ± S.E.M derived from three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with LPS stimulation (the 2nd lane).

3.3 The mechanism of Dioscorealide B and Dioscoreanone on NF-κB signaling pathway

3.3.1 The effect of Dioscorealide B and Dioscoreanone on the activation of IκBα protein in LPS-induced RAW 264.7 cells

The expression of iNOS IL-1β and IL-6 was majorly regulated through NF-κB signal transduction. NF-κB normally presents as an inactive form by coupling with IκBα (Whiteside and Israel, 1997). In the presence of LPS, IκBα is phosphorylated and degraded to release the active NF-κB. Then, the active NF-κB binds to κB-binding motif on the promoter of iNOS IL-1β and IL-6 genes (West et al., 2006). To determine the inhibitory effects of Dioscorealide B and Dioscoreanone on NF-κB activation, Western blot analysis on IκBα degradation and NF-κB-driven reporter assay were used in this section.

Upon exposure to LPS, IκBα was dramatically degraded within 20 min and then recovered to the normal level at 30 min after LPS stimulation. In Figure 25A, 30-minute pretreatment with Dioscorealide B inhibited LPS-induced IκBα degradation in a concentration-dependent manner. However, the inhibitory activity of Dioscorealide B at 6.0 μM could not recover the IκBα protein to the normal level. In Figure 25B, Dioscoreanone exerted the inhibitory activity on IκBα degradation in a concentration-dependent response. The IκBα protein could be reversed to resting level by Dioscoreanone at 2.5 and 5.0 μM. Data in this section may indicate that Dioscorealide B and Dioscoreanone inhibited the expression of iNOS, IL-1β and IL-6 mRNA through blocking IκBα degradation and inhibiting an active NF-κB release.

3.3.2 The effect of Dioscorealide B and Dioscoreanone on NF-κB binding activity in pNFκB-Luc-transfected RAW 264.7 cells

NF-κB is essential for the expression of various pro-inflammatory genes, including iNOS, IL-1β and IL-6 gene, which contain DNA binding sites for NF-κB protein in their promoter region (Siebenlist et al., 1994). To support the hypothesis that Dioscorealide B and Dioscoreanone inhibit IκBα protein degradation and further inhibit NF-κB binding activity, luciferase reporter system was analyzed. The *Cis*- NF-κB luciferase reporter system (pNFκB-Luc) encodes five repeated NF-κB binding consensus

sequences ([5'-TGGGGACTTTCCGC-3']$_5$) and fused to the firefly luciferase gene as a reporter gene.

In Figure 26, the pNFκB-Luc-transfected RAW 264.7 cells increased luciferase expression to 7.29 ± 0.51 -fold over the basal level upon exposure to LPS for 6 hrs. Pretreatment of Dioscorealide B and Dioscoreanone for 30 min inhibited luciferase activity. The percent inhibitions of Dioscorealide B (Figure 26A) were 28% and 45% at 3.0 and 6.0 μM, respectively. Dioscoreanone (Figure 26B) was significantly found to inhibit luciferase activity for 24% and 41% at 2.5 and 5.0 μM, respectively ($p < 0.05$).

The luciferase activity is depended on the expression of luciferase that is driven by a basic promoter element (TATA box) under the control of five repeated NF-κB binding motif element. The reduction of luciferase activity is corresponding to the inhibition of NF-κB binding activity. In this section, Dioscorealide B and Dioscoreanone inhibited LPS-induced luciferase activity. Therefore, this data suggests that both Dioscorealide B and Dioscoreanone inhibit NF-κB binding activity.

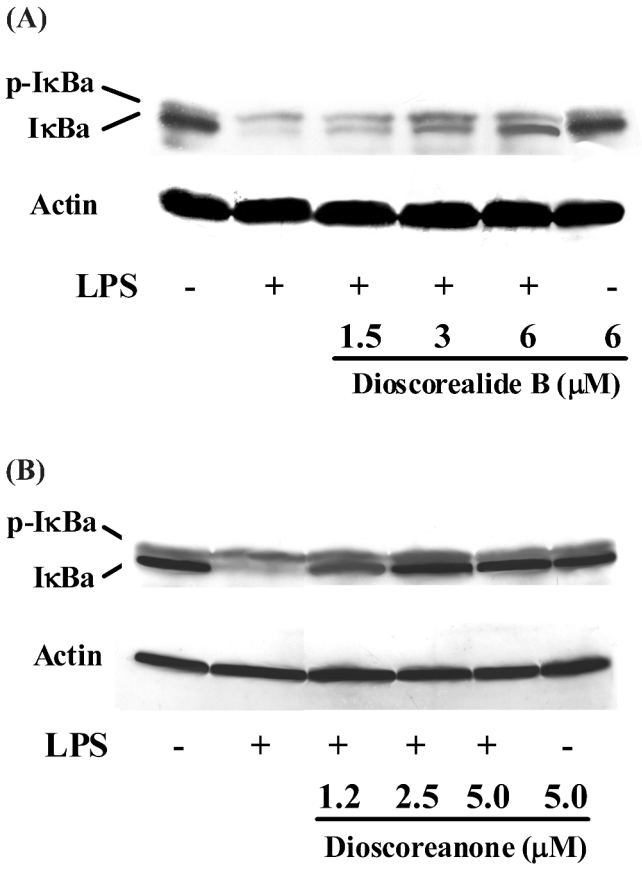

Figure 25. The effect of Dioscorealide B and Dioscoreanone on LPS-induced IκBα activation in RAW 264.7 macrophages

RAW 264.7 cells (10×10^6 cells/dish) were seeded in 10 cm-dish and allowed to adhere for 24 hrs. The adhered cells were pretreated with either **(A)** Dioscorealide B or **(B)** Dioscoreanone for 30 min followed by stimulation with LPS (2.5 μg/ml). After activation for 20 min, the total proteins were extracted and subjected to separate on 12% SDS-PAGE. The Western blot analysis was performed by anti-p- IκBα, anti-IκBα, or anti-actin antibody (internal control). These results represent one of the three independent experiments with similar results.

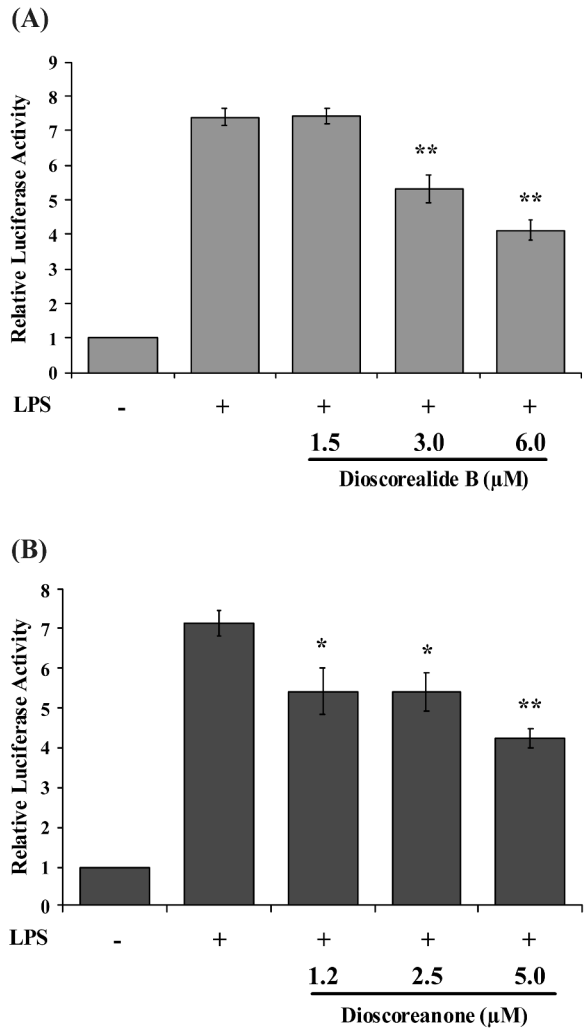

Figure 26. The effect of Dioscorealide B and Dioscoreanone on LPS-induced NF-κB-driven luciferase activity in pNFκB-Luc-transfected RAW 264.7 macrophages

RAW 264.7 cells (1×10^5 cells/well in 48-wells plate) were transfected with pNFκB-Luc plasmid for 24 hrs and then incubated with either **(A)** Dioscorealide B or **(B)** Dioscoreanone for 30 min followed by stimulation with 2.5 μg/ml LPS. After a 6-hour of activation, the total proteins were extracted and determined the luciferase activity. The relative light emission was expressed as a mean ± S.E.M derived from the three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with LPS stimulation (the 2nd bar).

3.4 The effect of Dioscorealide B and Dioscoreanone on the activation of ERK1/2 protein in LPS-induced RAW 264.7 cells

In section 3.2, IL-10 mRNA was suppressed by Dioscorealide B whereas Dioscoreanone did not exert this inhibition. The expression of IL-10 in the macrophages stimulated by LPS depends on the concomitant activation of MAPK proteins including ERK, p38 and JNK (Chanteux et al., 2007). MAPK protein family was normally activated by phosphorylation on their active sites. Therefore, the effect of Dioscorealide B and Dioscoreanone on the activation of ERK1/2 proteins was determined by Western blot analysis to verify phosphorylated ERK1/2 proteins (p-ERK1/2).

In Figure 27, the level of p-ERK1/2 was slightly detected in the resting state and increased upon exposure to LPS. Pretreatment with Dioscorealide B significantly reduced p-ERK1/2 level to baseline (Figure 27A). The suppressive phenomenon of Dioscorealide B was not due to the change of ERK1/2 total protein. In contrast, Dioscoreanone at 1.2 μM slightly suppressed p-ERK1/2 levels without significance (Figure 27B). At 2.5 and 6.0 μM of Dioscoreanone, the level of p-ERK1/2 proteins was rose higher than the LPS response. In addition, Dioscoreanone induced the up-regulation of p-ERK1/2 level (Figure 27B, lane 6) compared to the basal level (Figure 27B, lane 1).

This section showed that Dioscorealide B inhibited LPS-induced ERK1/2 activation. Dioscorealide B by itself did not alter ERK1/2 activation. In contrast, Dioscoreanone enhanced LPs-induced ERK activation with synergism. At 5.0 μM, Dioscoreanone could induce ERK1/2 activation by itself. These data support the hypothesis in section 3.2 that Dioscorealide B suppresses of IL-10 mRNA expression through the inhibition of ERK1/2 activation.

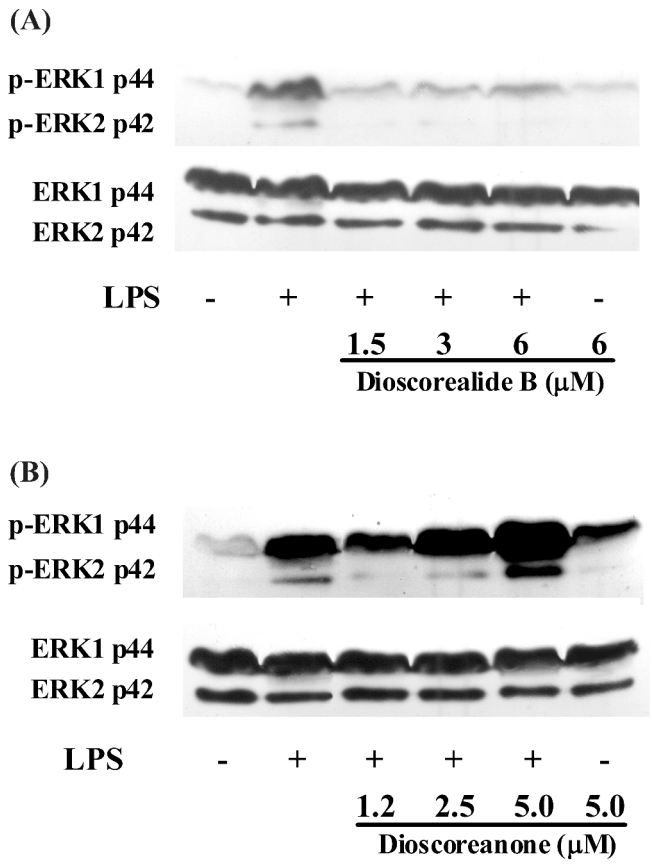

Figure 27. The effect of Dioscorealide B and Dioscoreanone on LPS-induced ERK1/2 activation in RAW 264.7 macrophages

RAW 264.7 cells (10×10^6 cells/dish) were seeded in 10 cm-dish and allowed to adhere for 24 hrs. The adhered cells were pretreated with either **(A)** 1.5-6.0 μM Dioscorealide B or **(B)** 1.2-5.0 μM Dioscoreanone for 30 min followed by stimulation with LPS (2.5 μg/ml). After 20-minutes of activation, the total proteins were extracted and processed by 12% SDS-PAGE. The Western blot analysis was performed by the anti-p-ERK1/2 antibody and anti-ERK1/2 antibody (internal control). These results represent one of three independent experiments.

3.5 The mechanisms of Dioscoreanone on the activation of ERK1/2 proteins

In section 3.4, Dioscoreanone by itself could induce ERK1/2 activation in RAW 264.7 cell. Dioscoreanone is 1,4 phenantraquinone (Itharat et al., 2003). Previously, Quinone compounds have been reported to activate MAPK proteins by either arylation or redox cycling reaction (Abdelmohsen et al., 2003). Therefore, this experiment aimed to evaluate the mechanism of Dioscoreanone on the activation of ERK1/2 proteins using Western blot analysis.

As shown in Figure 28A, ERK1/2 proteins were significantly activated by 1.2 - 5.0 μM of Dioscoreanone in a dose-dependent response. The maximal level of p-ERK1/2 proteins was found when RAW 264.7 cells were treated with 5.0 μM Dioscoreanone. The stimulation activity of Dioscoreanone on ERK1/2 proteins declined at 10 μM. In Figure 28B, 5.0 μM Dioscoreanone showed a time-dependent activation response. After exposure to 5.0 μM Dioscoreanone for 10 min, the phosphorylation level of ERK1/2 proteins significantly increased. The maximal levels of Dioscoreanone-induced ERK1/2 activation were maintained during 20 to 40 min and then declined at 80 min. Both dose- and time-dependent response of Dioscoreanone to ERK1/2 activation was not due to the significant change of the ERK1/2 proteins.

Quinone compounds can induce the activation of the MAPK signaling pathway by either the redox cycling reaction that is inhibited by Dicoumarol (Dic) (Klotz et al., 2002) or arylation to sulhydryl group that is inhibited by N-acetyl cysteine (NAC) (Abdelmohsen et al., 2003). To test the involvement of either the arylation or redox cycling reaction in the activation of ERK1/2, RAW 264.7 macrophages were pre-treated with NAC or Dic for 15 min and followed by stimulation with 5.0 μM Dioscoreanone. In Figure 29A, NAC or Dic alone did not affect the activation of ERK1/2 proteins (Figure 29A, lane 2 and 3 versus lane 1) whereas the level of p-ERK1/2 proteins increased in the presence of 5.0 μM Dioscoreanone (Figure 29A, lane 4 compared to lane 1). The Dioscoreanone-induced ERK1/2 activation was inhibited by Dic (Figure 29A, lane 5 versus lane 4). In contrast, NAC dominantly inhibited DN-induced ERK1 activation and slightly inhibited ERK2 activation (Figure 29A, lane 6 compare to lane 4).

The catalyzing groove of MAPK phosphatase has cysteine as an active site (Farooq and Zhou, 2004) that can be conjugated with quinone compound (Abdelmohsen et al., 2003), leading to the accumulation of the p-ERK1/2 proteins in cytoplasm.

Previous experiment (Figure 29A) indicated that the activation of ERK1/2 proteins by Dioscoreanone was affected on the arylation of sulhydryl group. To confirm this evidence, the time-course effect of LPS in the present of Dioscoreanone was determined. After LPS stimulation, ERK1/2 phosphorylation was observed at 10 min and declined at 50 min (Figure 29B, lanes 2-4). In the pre-treatment with Dioscoreanone for 30 min, the level of p-ERK1/2 increased at 10 and 20 min and decreased at 50 min after LPS stimulation (Figure 29B, lanes 5-7). At 50 min of LPS activation, the phosphorylated form of ERK1/2 in the presence of Dioscoreanone pre-treatment exerted more intensity than LPS alone (Figure 29B, lane 7 versus lane 4). When RAW 264.7 cells were co-cultured with Dioscoreanone and LPS for 20 min, the level of p-ERK1/2 protein was more intensity than LPS alone or Dioscoreanone alone (Figure 29B, lane 8 compared with lane 3 and lane 6).

This section showed that the activation of ERK1/2 proteins by Dioscoreanone was inhibited by NAC and Dic. The result indicates that Dioscoreanone induces ERK1/2 activation thought the arylating and redox cycling reaction liked the other Quinone compounds. This data was confirmed by the time-course response of LPS-induced ERK1/2 activation that was delayed in the presence of Dioscoreanone.

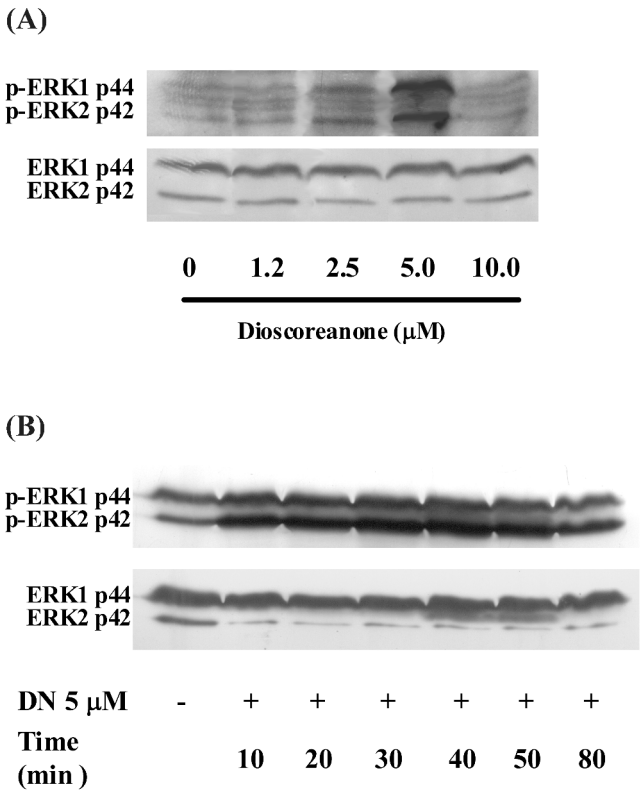

Figure 28. The effect of Dioscoreanone on ERK1/2 activation in RAW 264.7 macrophages

(A) RAW 264.7 macrophages (10×10^6 cells/dish) were seeded in 10 cm-dish and allowed to adhere for 24 hrs. The adhered cells were treated with the various concentrations of Dioscoreanone (DN) in the range of 1.2 -10.0 μM for 20 min. **(B)** RAW 264.7 cells were treated with 5.0 μM DN for the indicated times. The total proteins were extracted and processed by 12% SDS-PAGE. The Western blot analysis was performed by the anti-p-ERK1/2 antibody and anti-ERK1/2 antibody (internal control). These results represent one of three independent experiments.

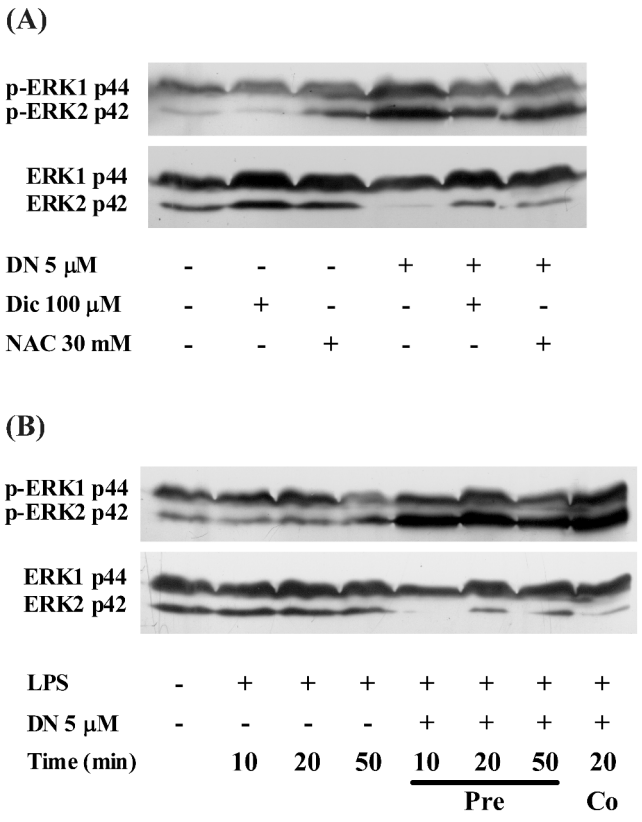

Figure 29. The effect of Dioscoreanone on the activation of ERK1/2 proteins in the presence of *N*-acetyl cysteine and Dicoumarol

(A) RAW 264.7 macrophages (10×10^6 cells/well) were seeded in 10 cm-dish and allowed to adhere for 24 hrs. The adhered cells were pretreated with either *N*-acetyl cysteine (NAC) or Dicoumarol (Dic) for 15 min prior to stimulation with Dioscoreanone (DN) for 20 min and then the total proteins were collected. **(B)** RAW 264.7 cells were treated with LPS in the absence of DN, 30-minute pretreatment (Pre) with DN or co-incubation (Co) with DN. The total proteins were extracted and processed by 12% SDS-PAGE. The Western blot analysis was performed by the anti-p-ERK1/2 antibody and anti-ERK1/2 antibody (internal control). These results represent one of three independent experiments.

CHAPTER 4

DISCUSSION

4.1 Anti-inflammatory activity of Dioscorealide B and Dioscoreanone

In the presence of inflammatory stimuli, the activation of pro-inflammatory signal transduction is triggered and produces inflammatory mediators such as cytokines, chemokines, lipid mediator and radical mediator. The up-regulated production of inflammatory mediators initiates and amplifies more complex inflammatory reactions such as the activation of inflammatory immune cells, the stimulated activity of enzymes in inflammatory response and neuro-physiological changes. The uncontrollable inflammatory mediator initiation leads to the progression of chronic diseases such as cardiovascular diseases, cancers, neurodegenerative disorders and local chronic inflammation (Pacher et al., 2007). Drugs that inhibit the production of inflammatory mediators or the activation of inflammatory signaling transduction may have beneficial therapeutic effects in the treatment of inflammatory-related disorders.

In this study, LPS-induced RAW 264.7 macrophage was used as the *in vitro* inflammatory model to evaluate the anti-inflammatory activity of natural compounds isolated from *Dioscorea membranacea*. Dioscorealide B and Dioscoreanone, the isolated compounds from *D. membranacea*, inhibited nitric oxide production without cytotoxicity (Figures 20 and 21). The inhibitory effect of Dioscorealide B and Dioscoreanone were due to the suppression of iNOS mRNA expression (Figures 23 and 24). Additionally, IL-1β and IL-6 mRNA expression were suppressed by Dioscorealide B and Dioscoreanone. Similarly, the anti-nitric oxide and TNF-α productions of *Dioscorea membranacea* extracts were demonstrated and the high active substances in these actions were Dioscorealide B Dioscoreanone and Diosgenin-3-*O*-α-rhamnosyl (1→2)-β-D-glucopyranoside (Tewtrakul and Itharat, 2007). Interestingly, Dioscorealide B inhibited the expression of IL-10 mRNA but Dioscoreanone did not.

Apart from maintaining normal physiological function, nitric oxide is required in the regulation of diverse physiological processes including smooth muscle contractility, platelet reactivity, neurotransmission and the cytotoxic actions of the immune system. Inappropriate release of nitric oxide has been linked to a number of pathologies.

Nitric oxide acts as a secondary messenger to initiate guanylate cyclase within vascular smooth muscle to produce cyclic Guanosine Monophosphate (cGMP) which allows blood vessels to relax and increases blood flow (Ignarro et al., 1986). The relaxation of blood vessels and increase in blood flow lead to hyperemia, stasis and increased vascular permeability during acute inflammatory response (Chandrasoma and Taylor, 1998). In addition, nitric oxide can interact with Reactive Oxygen Species (ROS) to form peroxynitrite (Koppenol, 1998) which inhibits enzyme function and induces DNA damage, lipidperoxydation and cell death (Wink and Mitchell, 1998). Besides, the increased levels of nitric oxide and peroxynitrite return to directly induce the expression and the activity of COX that lead to increase the production of arachidonic acid metabolites (Mollance et al., 2005). The high production of arachidonic metabolites such as prostaglandins mediates a number of cardinal signs during acute inflammation, including fever, pain and vascular permeability (Rosenberg and Gallin, 2003).

IL-1β mediates a wide variety of biological effects during inflammation (Durum et al., 1990). Endothelial cells are induced by IL-1β to increase their expression of ligands for integrins, including ICAM-1 and VCAM-1. ICAM-1 binds to the integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), whereas VCAM-1 binds to the integrins LFA-4. These ligands participate in adhesion, arrest and transmigration of leukocytes. Macrophages that are exposed to IL-1β, increase the production of prostaglandin, cytokine, ROS and RNS. The increase of inflammatory mediators by macrophages amplifies the progression of inflammation, resulting in the implication of the etiology of some chronic inflammatory diseases. In addition, IL-1β induces the hypothalamus to increase the body temperature and stimulates the liver in the synthesis of acute-phase protein (Kumar et al., 2007).

The best correlations of clinical severity in inflammatory, autoimmune or infectious diseases with plasma cytokine levels have been made with IL-6. IL-6 is a pleiotropic cytokine that modulates the immune system and inflammation (Rose-John et al., 2006). IL-6 directs T cell recruitment by regulating local chemokines secretion and chemokines receptor expression. On cell differentiation, IL-6 directs monocytes away from dendritic lineage to a macrophage phenotype, and blocks the immunosuppressive activity of regulatory T lymphocytes. In addition, IL-6 stimulates hepatocytes to produce acute-phase proteins and it causes leukocytosis and fever (Naka et al., 2002). The accumulation of leukocytes, differentiation of monocytes, activation of hepatocytes and inhibition of

regulatory T cell activity are implicated in the disease progression of many diseases such as arthritis, cancer and chronic inflammation. Therefore, this study showed that Dioscorealide B and Dioscoreanone suppressed the expression of IL-6 mRNA. It can be implied that Dioscorealide B and Dioscoreanone exert a potency to treat chronic and systemic inflammatory conditions. In Figures 23 and 24, the level of IL-6 was reduced by the Dioscorealide B and Dioscoreanone.

Unlike IL-1 and IL-6, IL-10 is a cytokine synthesis inhibitory factor which is mostly produced by T cells and minor by monocyte/macrophages. The production of IL-10 affects numerous cell populations (Moore et al., 2001). The inflammatory mediators that are produced from many kinds of inflammatory cells such as monocytes/macrophages, neutrophils, eosinophils, dendritic cells and lymphocytes are inhibited by the response of IL-10 activity. These mediators are mostly presented in both acute and chronic inflammation such as TNF, IL-1, IL-6, IL-8, chemokines, nitric oxide and ROS. Although Dioscorealide B inhibited the expression of IL-10 mRNA, the IL-10 receptor does not respond to IL-10 in some chronic inflammations (Avdiushko et al., 2001).

4.2 The mechanism of Dioscorealide B and Dioscoreanone on LPS-induced macrophages

4.2.1 The effect of Dioscorealide B and Dioscoreanone on NF-κB activation

In Section 3.2, it was demonstrated that Dioscorealide B and Dioscoreanone inhibited iNOS, IL-1β and IL-6 but not IL-10 mRNA expression. The expression of iNOS, IL-1β , IL-6 and IL-10 is under the control of NF-κB binding motif on their promoter region. The mouse iNOS promoter regions contain two sites of NF-κB binding motif upstream of the TATA box (Xie et al., 1994), including -85 to -76 (GGGACTCTCC) and -971 to -962 (GGGATTTTCC). The -300 region of IL-1β promoter contains a functional NF-κB binding motif composed of decamer sequence 5'-GGGAAAATCC-3' (Hiscott et al., 1993). At -72 to -62 of IL-6 promoter, the NF-κB binding motif presents as the sequence 5'-GGGATTTTCC-3' (Zhang et al., 1990). In addition, IL-10 also contains NF-κB binding motif at -55 to -46 (GGAAGGCCCC) but this sequence responds to p50 homodimer (Cao et al., 2006). The κB-binding consensus sequences in reporter system are 5'-TGGGGACTTTCCGC-3'. It is possible that

Dioscorealide B and Dioscoreanone inhibit iNOS, IL-1β, IL-6 and IL-10 through the suppression of promoter region.

Using the NF-κB-driven Luciferase reporter system, Dioscorealide B and Dioscoreanone reduced Luciferase activity, meaning that the binding activity of NF-κB was inhibited by these two compounds (Figure 25). NF-κB normally presents as an inactive form by coupling with IκBα (Whiteside and Israel, 1997). In the presence of LPS, IκBα is phosphorylated and degraded to release the active NF-κB. Then, the active NF-κB binds to κB-binding motif on the promoter of iNOS IL-1β and IL-6 genes (West et al., 2006). The binding activity of NF-κB is controlled by the phosphorylation and degradation of IκB protein. The inhibition of Dioscorealide B and Dioscoreanone resulted in the inhibition of IκBα degradation. In addition, treatment with either Dioscorealide B or Dioscoreanone did not alter the levels of p-IκBα protein, which were present at basal levels (Figure 26, the upper bands). This result implies that the inhibitory effect of Dioscorealide B and Dioscoreanone affects the upstream signaling pathway of IκBα activation, such as the IKK proteins, because the level of p-IκBα should increase in the presence of a downstream inhibitor such as a proteasome inhibitor (Takeda et al., 2003). Data in Section 3.3 indicate one of the possibilities that Dioscorealide B and Dioscoreanone inhibit LPS-induced IκBα degradation through inhibition of the up-stream IKK proteins, leading to the inactivation of NF-κB proteins and the down-regulation of transcriptional genes including iNOS, IL-1β and IL-6 during LPS-induced RAW 264.7 murine macrophages.

4.2.2 The effect of Dioscorealide B and Dioscoreanone on ERK1/2 activation

Although the LPS-induced NF-κB activation of Dioscoreanone-treated cells was inhibited, the IL-10 mRNA expression could be induced in the same condition (Figures 23 and 24). This result indicates either NF-κB signaling pathway is not essential for IL-10 regulation or Dioscoreanone inhibits p50 homodimer in another pathway. In the murine B-cell lymphoma cell line, the activation of p50 homodimer is controlled by the activation of the MEK/ERK pathway (Kurland et al., 2003). Moreover, the Sp1 transcription factor which is essential for IL-10 production is regulated by MAPK signaling, especially p38 and ERK1/2 MAPKs (Chanteux et al., 2007). Interestingly, Denbinobin (a natural occurring 1,4-phenantrenequinone) which displays a chemical structure like Dioscoreanone (1,4-phenantraquinone) has been reported to inhibit NF-κB

activation without the alteration of Sp1 binding activity (Sanchez-Duffhues et al., 2008). It can be hypothesized that Dioscorealide B and Dioscoreanone exert a different role in the suppression of IL-10 expression through the activation of ERK1/2 proteins.

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that are a part of the signal transduction pathways, which connect the extracellular signal of LPS to intracellular responses in macrophages (Lee and Kin, 2007). p38 MAPK and JNK are members of the MAPK family which regulate inflammatory proteins as well as immune responses and expression of various pro-inflammatory mediators, e.g., TNF-α, IL-1β, IL-6 and iNOS (Hommes et al., 2003) while ERK members exert anti-inflammatory signaling in macrophages (Watters et al., 2002) by inducing the expression of IL-10 (Chanteux et al., 2007). The activation of ERK1/2 may induce the activation of MAPK phosphatase 1 (MKP-1). Then, MKP-1 reduces TATA binding protein (TBP) activity which associates with active NF-κB on the promoter region and enhances the transcriptional activity of NF-κB (Chen et al., 2002; Xiao et al., 2002). In this study, Dioscorealide B inhibited LPS-induced ERK1/2 activation (Figure 27A) but Dioscoreanone enhanced the up-regulation of p-ERK1/2 proteins (Figure 27B). Dioscoreanone in the absence of LPS induced the activation of ERK1/2 proteins (Figure 27B, lane 6), whereas Dioscorealide B alone did not affect p-ERK1/2 proteins (Figure 27A, lane 6). These results may imply that Dioscorealide B inhibits LPS-induced ERK1/2 activation, leading to the inhibition of IL-10 mRNA expression. In contrast, Dioscoreanone enhanced the up-regulation of ERK1/2 activation, leading to the maintaining of IL-10 mRNA expression. The enhancement of ERK1/2 by Dioscoreanone may consequently inhibit NF-κB binding activity caused by the activation of MKP-1 and induce the inhibition of the activity of TBP (Carter et al., 1999).

Data in this section imply different actions of Dioscorealide B and Dioscoreanone on IL-10 mRNA expression. Dioscorealide B inhibited both the IκBα phosphorylation and ERK1/2 activation pathways, whereas Dioscoreanone inhibited only NF-κB activation. Focusing on the stimulation of macrophages, LPS induces the activation of a protein cascade through TLR4-CD14-MD-2 complex (Takeda et al., 2003), which regulates downstream kinase signal including IRAK4, PI3K, PKR and Ras (Guha and Mackman, 2001; West et al., 2006). It can be hypothesized that the inhibitory effect of Dioscorealide B may affect the protein kinases more than the activation of IKKs and

MEK1/2 proteins. Additionally, the natural compounds may act as non-specific kinase inhibitors in the same fashion as Quercetin (Davies et al., 2000).

4.2.3 The influence of Dioscoreanone on the activation of ERK1/2 proteins

Dioscoreanone alone induced the activation of ERK1/2 proteins in the absence of LPS in both dose- and time-response trials (Figure 28). This mechanism of Dioscoreanone may involve a 1, 4 dione structure in its molecule as a functional group. From the literature, Quinone compounds such as Meinadione (2-methyl-1, 4 naphthoquinone or Vitamin K_3), *p*-Benzoquinone (1, 4 benzoquinone) and Dimethoxy Napthoquinone (2, 3-dimethoxy-1, 4-naphthoquinone) have been found to induce ERK1/2 activation by two pathways. The first one depends on arylation with the nucleophillic thiol group (sulhydryl group: -SH) leading to inactivation of the protein phosphatases and increases in intracellular oxidative stress by reducing the intracellular anti-oxidant glutathione (GSH) (Abdelmohsen et al., 2003). The second pathway, also called redox cycling, is elicited by NAD(P)H: quinone oxidoreductase (NQOR), which consequently produces the superoxide anion which activates the phosphorylation of ERK1/2 proteins (Klotz et al., 2002).

Using *N*-acetyl cysteine and Dicoumarol as an inhibitor for the arylation and redox cycling reactions, the activation of the ERK1 protein by Dioscoreanone was inhibited by both Dicoumarol and *N*-acetyl cysteine, whereas the activation of ERK2 protein was only suppressed by *N*-acetyl cysteine (Figure 29A). This suggests that the mechanism of Dioscoreanone on the activation of ERK1 was mediated by stress induction elicited through the activity of NQOR and arylation with the sulhydryl group. In contrast, the activation of ERK2 by Dioscoreanone was mediated through only a redox cycling reaction that increased superoxide anions and oxidative stress. In addition, the Quinone compounds bind to critical cysteine residues in the active site of protein tyrosine phosphatases (PTPase) and inhibit their function (Abdelmohsen et al., 2003). As shown in Figure 29B, the decline phase of LPS-induced ERK1/2 activation was delayed in the pretreatment stage with Dioscoreanone. This suggests that Dioscoreanone has the ability to conjugate a sulhydryl amino acid cysteine in the catalytic grove of phosphatase (Farooq and Zhou, 2004), leading to the inhibition of phosphatase activity and the accumulation of active ERK1/2 proteins. Moreover, the generation of oxidative stress by Dioscoreanone

also inhibits IκBα degradation and suppresses the LPS-stimulated NF-κB binding activity in similar fashion to that found in hydrogen peroxide treatment (Torrie et al., 2001). This data indicates that Dioscoreanone induces ERK1/2 activation through two mechanisms. First, Dioscoreanone acts as NQOR substrate to initiate ROS that lead to activate ERK1/2 phosphorylation. And, the sulhydryl arylation by Dioscoreanone leads to inhibit either Protein tyrosine phosphatase activity or Thiol-antioxidant system leading to oxidative stress induction. The overall of the proposed mechanism of Dioscorealide B and Dioscoreanone are shown in Figure 30.

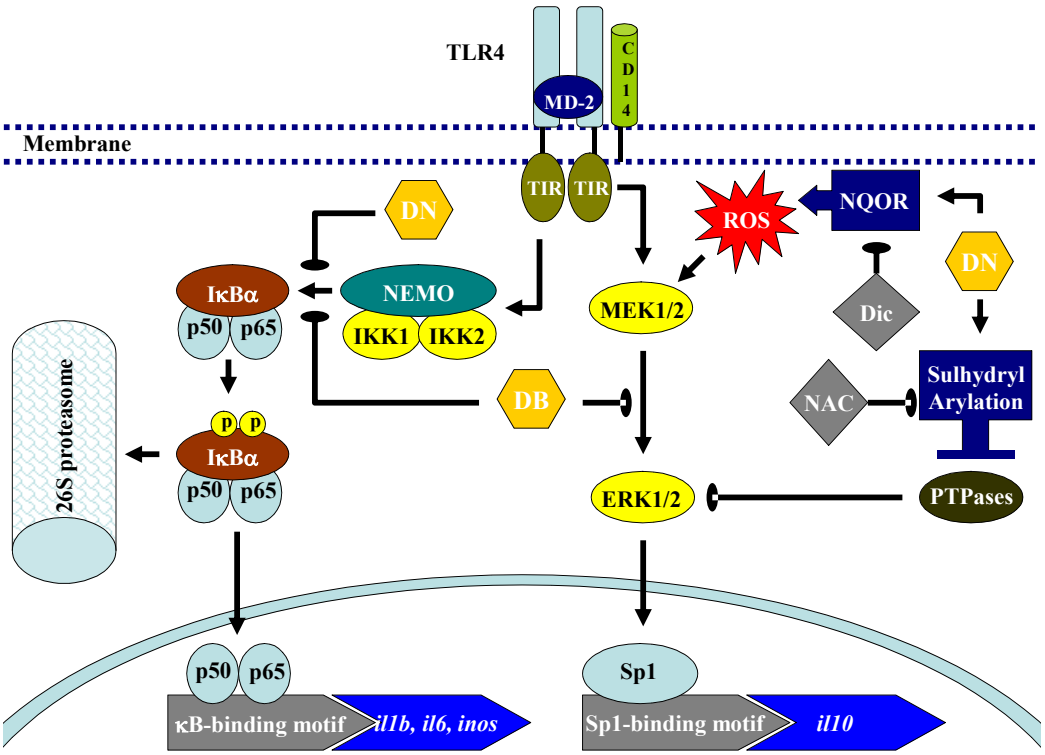

Figure 30. The proposed mechanisms of Dioscoreanone B and Dioscoreanone on LPS-induced RAW 264.7 macrophages.

CHAPTER 5

CONCLUSIONS

1. The effects of Dioscorealide B on LPS-induced RAW264.7 macrophages

Dioscorealide B inhibits nitric oxide release in a concentration-dependent manner with IC_{50} equal to 2.85 ± 0.62 μM. The inhibition of nitric oxide release is not due to the cytotoxicity of Dioscorealide B. The suppression of nitric oxide release in a 24-hour period of LPS stimulation is reduced when Dioscorealide B is added 3 hrs later than LPS. iNOS, IL-1β, IL-6 and IL-10 mRNA expression are suppressed by Dioscorealide B in a concentration-dependent manner. Dioscorealide B inhibits NF-κB binding activity in pNFκB-Luc-transfected RAW264.7 macrophages. Using Western blot analysis, Dioscorealide B inhibits both IκB degradation and ERK1/2 activation.

2. The effects of Dioscoreanone on LPS-induced RAW264.7 macrophages

Dioscoreanone inhibits nitric oxide release in a concentration-dependent manner with IC_{50} equal to 2.50 ± 0.64 μM. The inhibition of nitric oxide release is not due to the cytotoxicity of Dioscoreanone. The suppression of nitric oxide release in a 24-hour period of LPS stimulation is reduced when Dioscoreanone is added at 3 hours later than LPS. iNOS, IL-1β and IL-6 mRNA expression are concentration-dependently reduced in the presence of Dioscoreanone but the expression of IL-10mRNA is not modulated. Dioscoreanone inhibits NF-κB binding activity in pNFκB-Luc-transfected RAW264.7 macrophages. Using Western blot analysis, Dioscoreanone B inhibits IκB degradation and supports LPS-induced ERK1/2 activation. Dioscoreanone stimulates ERK1/2 phosphorylation in a concentration- and time-dependent manner. The activation of ERK1/2 by Dioscoreanone is inhibited by *N*-acetyl cysteine and Dicoumarol.

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APPENDIX A

ADDITIONAL INFORMATIONS

A-1). The purification of Dioscorealide B and Dioscoreanone

The rhizomes of *Dioscorea membranacea* (Pierre ex Prain & Burkill) were collected from Chumporn province, Thailand. Voucher specimens were deposited at the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Dried ground rhizomes (1 Kg) were percolated with 95% ethanol, and then dried by an evaporator under reduced pressure. The percent yield of ethanol extract was 3.1%.

In Figure 31, the ethanol extract (10 g) was fractionated by Vacuum Liquid Chromatography (VLC) using chloroform, chloroform-methanol (1:1) and methanol as eluting solvents. Fraction A (chloroform elution) was purified by silica gel column chromatography using gradient eluting solvent (hexane, chloroform and methanol) to obtain Dioscoreanone from fraction A3 and Dioscorealide B from fraction A4. The NMR spectrums were shown in Table 18.

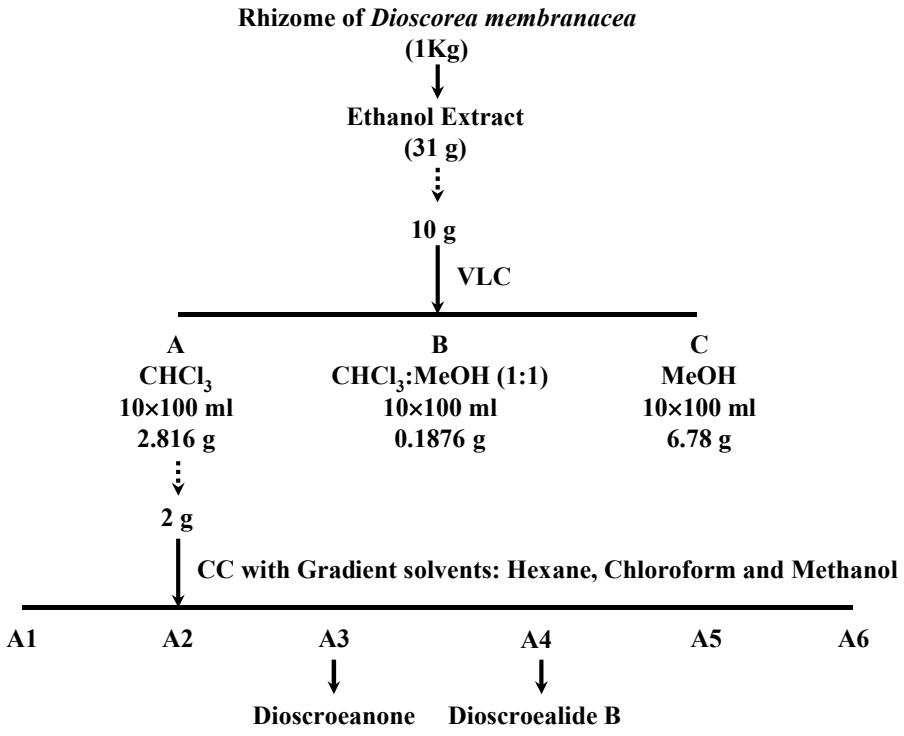


Figure 31. The flowchart shows the processes to purify Dioscorealide B and Dioscoreanone from the rhizomes of *Dioscorea membranacea*

Table 18. NMR spectrum (500 MHz for ^{1}H and 125 Hz for ^{13}C) of Dioscoreanone and Dioscorealide B in CD_3Cl_3 (Itharat et al., 2003)

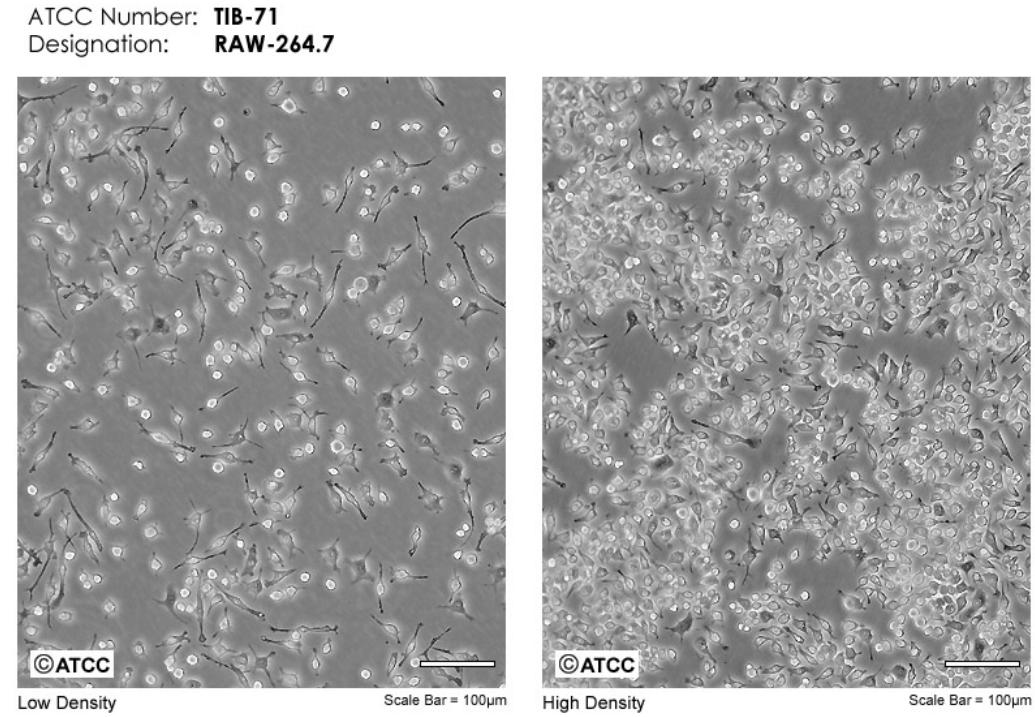
Dioscorealide B			Dioscoreanone		
Carbon Position	δ_C (mult.)	δ_H (mult., *J* in Hz)	Carbon Position	δ_C	δ_H (mult., *J* in Hz)
1	168.9 (s)		1	181.4	
1'	114.9 (s)		2	158.6	
1"	136.8 (s)		3	111.3	6.11 (s)
2	121.2 (d)	7.76 (d, 8.5)	4	189.3	
3	128.2 (d)	7.8 (d, 8.5)	4'	125.7	
3'	138.5 (s)		4"	126.3	
3"	116.3 (s)		5	106.4	9.13 (s)
4	103.9 (d)	7.36 (d, 2.5)	6	151.6	
5	162.2 (s)		7	149.0	
6	110.7 (s)	6.88 (d, 2.5)	8	110.1	7.24 (s)
6'	152.1 (s)		8'	135.1	
8	94.1 (d)	6.03 (s)	9	132.3	7.92 (d, 8.5)
8'	130.7 (s)		10	121.0	8.06 (d, 8.5)
9	141.9 (s)		10'	129.4	
5-OMe	56.1 (q)	3.96 (s; 3H)	2-OMe	56.3	3.93 (s; 3H)
9-OMe	60.0 (q)	4.13 (s; 3H)	6-OMe	56.1	4.12 (s; 3H)

A-20). Description of RAW 264.7 macrophages (TIB-71: ATCC)

Designation	RAW 264.7
Depositor	W.C. Raschke
Organism	*Mus musculus* (mouse)
Strain	BALB/c
Gender	male
Age	adult
Tissue	ascites
Disease	Abelson murine leukemia virus-induced tumor
Morphology	Monocyte/macrophage Abelson murine leukemia virus-transformed cell
Growth Property	adherent
Antigen Expression	H-2d
Cellular Products	lysozyme
Receptors expressed	complement (C3)
Comments	This line was established from a tumor induced by Abelson murine leukemia virus. They are negative for surface immunoglobulin (sIg-), Ia (Ia-) and Thy-1.2 (Thy-1.2). This line does not secrete detectable virus particles and is negative in the XC plaque formation assay. The cells will pinocytose neutral red and will phagocytose latex beads and zymosan. They are capable of antibody dependent lysis of sheep erythrocytes and tumor cell targets. LPS or PPD treatment for 2 days stimulates lysis of erythrocytes but not tumor cell targets.
Propagation	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** 95% air and 5% carbon dioxide (CO_2) **Temperature:** 37.0 °C

Subculturing	**Protocol:** Subcultures are prepared by scraping. For a 75 cm^2 flask, remove all but 10 ml culture medium (adjust amount accordingly for other culture vessels). Dislodge cells from the flask substrate with a cell scraper; aspirate and add appropriate aliquots of the cell suspension into new culture vessels.
	Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended
	Medium Renewal: Replace or add medium every 2 to 3 days.
Preservation	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO
	Storage temperature: liquid nitrogen vapor phase

(A)



(B) **(C)**

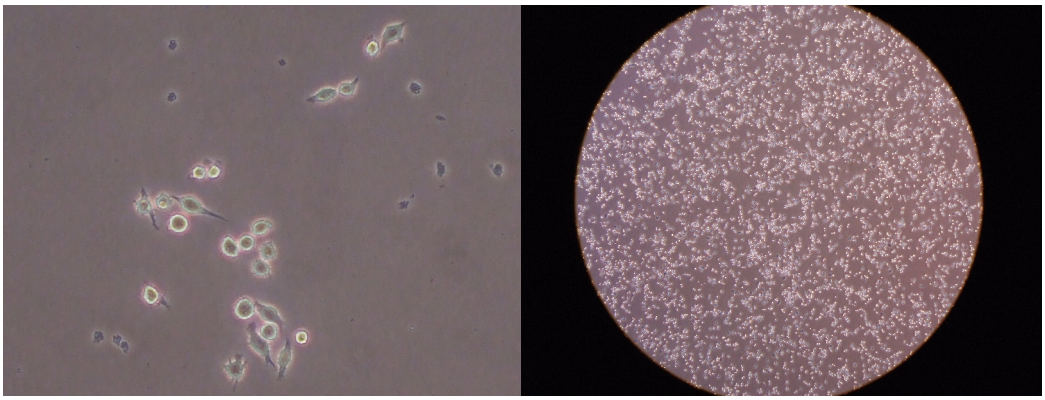


Figure 32. The illustrations demonstrate the morphology of RAW 264.7 macrophages under reverted light microscope

(A) The low density and the high density of RAW 264.7 cell (ATCC). **(B)** the morphology of RAW 264.7 cells in high power field. **(C)** 60% confluent RAW 264.7 macrophages in low power field.

APPENDIX B

STOCK SOLUTIONS AND REGENTS

B-1). Stock solutions of isolated compounds

1). 8 mM Dioscorealide B

Dioscorealide B (2.4 mg, FW = 300) is dissolved in 1 ml of DMSO and incubated at room temperature overnight. Then, stock solution was kept at -20 °C.

2). 5 mM Dioscoreanone

Dioscoreanone (2.8 mg, FW = 284) is dissolved in 2 ml of DMSO and incubated at room temperature overnight. Then, stock solution was kept at -20 °C.

B-2). Cell culture reagents

1). Complete Dulbecco's Modified Eagle Medium (DMEM)

DMEM powder (Gibco), 1.52 g Sodium bicabornate (Sigma) and 10 ml 10000 unit/ml penicillin-streptomycin (Gibco) are dissolved in 500 ml sterile distilled water, adjusted pH to 7.2 and added up with sterile distilled water to 1000 ml. DMEM-0 solution is passed trough 0.2-μm filter to sterilize and stored in 200 ml aliquots at -20 °C. Fetal bovine serum or FBS (Biochrom AG) is passed trough 0.2-μm filter to sterilize and stored in 100 ml aliquots at -20 °C. Complete DMEM is prepared by adding 20 ml FBS to 200 ml DMEM and stored at -20 °C.

2). 10× Phosphate Buffer Saline (10× PBS)

Sodium dihydrogen phosphate or NaH_2PO_4 (0.23 g), Disodium hydrogen phosphate or Na_2HPO_4 (1.15 g) and Sodium chloride or NaCl (9.0 g) are dissolved in distilled water, adjusted pH to 7.2 and add up to 100 ml. This solution is autoclaved to

sterile and stored at room temperature. 1× PBS is prepared by diluting 10 ml 10× PBS with 90 ml distilled water and autoclaving to sterile. 1× PBS is stored at 4 °C.

B-3). Stock solutions of stimulator and inhibitors

1). 2.5 mg/ml Lipopolysaccharide (*E. coli* O111:B4)

Lipopolysaccharide from *E. coli* O111:B4 (25 mg) is dissolved in 10 ml of sterile distilled water and incubated at room temperature overnight. Then, stock solution was passed trough 0.2-μm filter to sterilize and kept at -20 °C.

2). 150 mM Dicoumarol (Dic)

3, 3'-methylenl-bis (4-hydroxy coumarin) or Dicoumarol (0.0504 g, FW = 336.3) is dissolved in 1 ml 0.1 N NaOH and passed trough 0.2-μm filter to sterilize. This solution is stored at 4 °C.

3). 600 mM *N*-acetyl cysteine (NAC)

N-Acetyl-L-cysteine (0.9792 g, FW = 163.2) is dissolved in 1× PBS, adjusted pH to 7.2 and added up with 1× PBS to 10 ml. This solution is passed trough 0.2-μm filter to sterilize and stored at 4 °C.

B-4). Griess's reagents

1). NED solution (0.2% *N*-1-napthylethelenediamine dihydrochloride)

N-1-napthylethelenediamine dihydrochloride (0.1 g) is diluted in 50 ml deionized water. The reagent was stored at 4 °C protected from light.

2). Sulfanilamide Solution (2% Sulfanilamide)

Sulfanilamide (1.0 g) is prepared with 6 ml o-phosphoric acid and 44 ml deionized- water. The reagent was stored at 4 °C protected from light.

3). Standard Sodium Nitrite (100 mM)

$NaNO_2$ (0.069 g, FW = 69.00) is dissolved in 10 ml deionized water. The reagent was stored at 4 °C protected from light.

B-5). MTT assay reagent: 10× MTT stock solution (5 mg/ml)

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (25 mg) is dissolved in 5 ml of PBS or normal saline and filtered with 0.2 μm to sterile. Reagent is kept at -20 °C and protected from light. Working MTT solution is 1× MTT solution (0.5 mg/ml) in DMEM-10.

B-6). RT-PCR reagents

1). 500 μg/ml Oligo (dT)$_{16}$

The lyophilized Oligo (dT)$_{16}$ (100 μg/tube) is dissolved in 200 μl DEPC-treated water. This reagent is kept at -20 °C.

2). 100 mM Primers (10× Primer)

100 mM Primers are prepared from stock lyophilized primers (Invitrogen) by adding sterile deionized water.

B-7). Agarose gel electrophoresis reagents

1). 0.5 M EDTA, pH 8.0

EDTA (18.61 g, FW = 372.24) is dissolved in 80 ml distilled water. The solution is adjusted the pH to 8.0 and brought up to 100 ml.

2). 5× TBE buffer

Tris base (54 g, FW = 121.1), Boric acid (27.5 g, FW = 61.83) and 20 ml 0.5 M EDTA (pH 8.0) are dissolved in distilled water and brought up to 1000 ml. The working solution is 0.5× buffer that is prepared by diluted 100 ml 5× buffer with 900 ml distilled water.

3). 10 mg/ml Ethidium bromide

Ethidium bromide (1 g) is dissolved in 100 ml distilled water and stir on a magnetic stirrer for several hours to ensure that the dye has been dissolved well. Dye solution is protected from light and stored at room temperature.

4). 6× Gel loading dye

Glycerol (1.25 ml), 0.5 M EDTA (0.6 ml) and Bromphenol blue (12.5 mg) are dissolved in 5 ml distilled water. The solution is kept at -20 °C.

B-8). SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot reagents

1). 1.5 M Tris, pH 8.8

Tris (hydroxymethyl) aminomethane (9.08 g, FW = 121.1) is dissolved in 30 ml distilled water. The solution is adjusted pH to 8.8 and brought up to 50 ml. The stock solution is autoclaved and kept at 4 °C.

2). 1.0 M Tris, pH 6.8

Tris (hydroxymethyl) aminomethane (6.06 g, FW = 121.1) is dissolved in 30 ml distilled water. The solution is adjusted pH to 6.8 and brought up to 50 ml. The stock solution is autoclaved and kept at 4 °C.

3). 10% Sodium Dodesyl Sulfate (SDS)

SDS (5 g) is dissolved in 50 ml distilled water. The stock solution is kept at 4 °C.

4). 10% v/v Ammonium persulfate (APS)

Ammonium persulfate (0.1 g) is dissolved in 1 ml distilled water. This solution is kept at 4 °C for a month.

5). 4× Protein loading dye

Dithiohtreitol or DTT (3.085 g), 0.2 g Bromophenol blue, 4 ml 2-β-mercaptoethanol, 20 ml Glycerol, 4 g SDS and 10 ml 1 M Tris (pH 6.8) are dissolved in distilled water and brought up to 50 ml. The solution is stored in 0.5 ml aliquots at -70 °C.

6). 10× Running buffer

Tris (hydroxymethyl) aminomethane (30.28 g, FW = 121.1), Glycine (144.13 g, FW = 75.07) and SDS (10 g) are dissolved in 1000 ml distilled water. The stock solution is kept at 4 °C. For 1× working solution, 100 ml 10× Running buffer is diluted with 900 ml distilled water and kept at 4 °C.

7). 10× Transfer buffer

Tris (hydroxymethyl) aminomethane (15.15 g, FW = 121.1) and Glycine (72 g, FW = 75.07) are dissolved in 500 ml distilled water. For 1× working solution, 100 ml 10× Transfer buffer and 200 ml absolute Methanol are diluted with 700 ml distilled water and kept at 4 °C.

8). 2 M Tris, pH 8.0

Tris (hydroxymethyl) aminomethane (121.1 g, FW = 121.1) are dissolved in distilled water, adjusted pH to 8.0 and brought up to 500 ml. The stock solution is autoclaved and kept at 4 °C.

9). 10× Tween-20 TBS buffer (TTBS)

Tween-20 (5 ml), Sodium chloride (45 g) and 50 ml 2 M Tris, pH 8.0 are dissolved in 500 ml distilled water. The stock solution is kept at 4 °C. For 1× working solution, 100 ml 10× TTBS is diluted with 1000 ml distilled water and kept at 4 °C.

10). Stripping buffer

2-Mercaptoethanol (1.4 ml), 40 ml 10% SDS and 12.5 ml 1 M Tris, pH 6.8 are dissolved in sterile distilled water and added up to 200 ml. This solution is stored at room temperature.

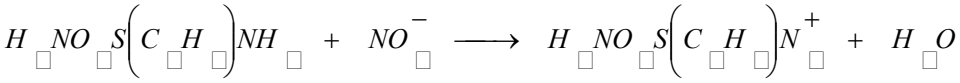
APPENDIX C

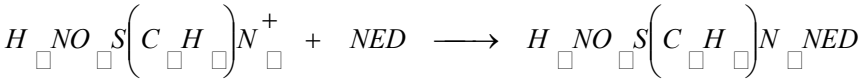
ASSAY PROTOCOLS

C-1). Nitrite concentration determination using Griess's reaction

1). Principle

The Griess Reagent system is based on the chemical reaction shown below, which uses sulfanilamide and *N*-1-napthylethelenediamine dihydrochloride (NED) under acidic (phosphoric acid) condition. This system detects nitrite (NO_2^-) in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium. The nitrite sensitivity is dependent on the matrix. The limit of detection is 2.5 μM nitrite (in ultra pure deionized distilled water) using this protocol.





2). Reagents (Appendix B-6)

1. NED reagent
2. Sulfanilamide reagent
3. Standard sodium nitrite 100 mM

3). Equipment

1. Micro plate reader BIO RAD model 550 using wavelength at 570 nm filter

4). Nitrite Measurement

4.1). Preparation of a Nitrite Standard Reference Curve

For each assay, sodium nitrite standard curve should be prepared for accurate quantification of nitrite level in experimental samples.

1. Prepare 1 ml of 100 μM nitrite solution by diluting 100 mM nitrite standard 1: 1000 in complete DMEM.

2. Designate 3 columns (column 1-3) in the 96-well plate for the nitrite standard reference curve. Dispense 100 μl of complete DMEM into the wells in rows B-H.

3. Add 200 μl of the 100 μM nitrite solution to the remaining 3 wells in row A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 μM $NaNO_2$			**Sample**								
B	50 μM $NaNO_2$											
C	25 μM $NaNO_2$											
D	12.5 μM $NaNO_2$											
E	6.25 μM $NaNO_2$											
F	3.13 μM $NaNO_2$											
G	1.56 μM $NaNO_2$											
H	0 μM $NaNO_2$											

4. immediately perform 6 serial two-fold dilution (100 μl/well) in triplicate down the plate to generate the nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μM), discarding 100 μl from the 1.56 μM set of wells. Do not add any nitrite solution to the last set of wells (0 μM)

4.2). Nitrite measurement

1. Allow the Sulfanilamide solution and NED solution to equilibrate to room temperature (15-30 min).

2. Add 100 μl of each experimental sample to wells in triplicate.

3. Using a multi-channel pipette, dispense 50 μl Sulfanilamide solution to all experimental samples and wells containing the dilution series for the Nitrite standard curve.

4. Incubate 15 min at room temperature, protected from light.

5. Using a multi-channel pipette, dispense 50 μl the NED solution to all wells.

6. Incubate 15 min at room temperature, protected from light. A purple/magenta color will begin to form immediately.

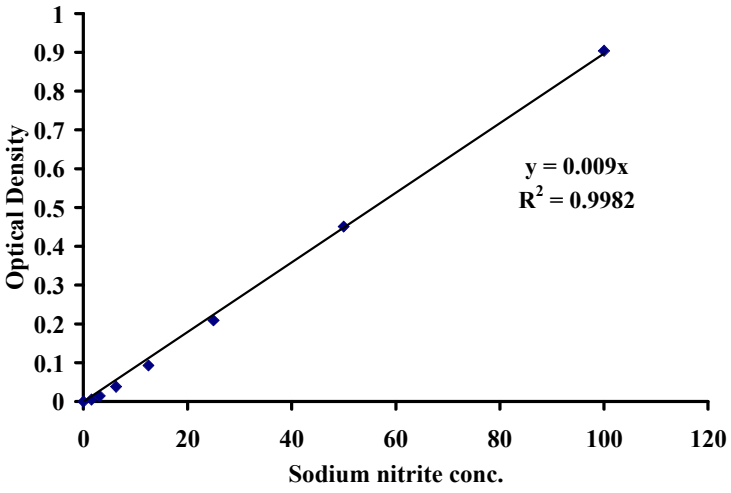
7. Measure absorbance within 30 min in micro-plate reader with a 570 nm filter.

8. Determine nitrite concentration by using nitrite standard reference curve (Figure 33).

9. The percentage of inhibition is calculated by mean nitrite concentration of triplicate wells using equation:

$$\% inhibition = \frac{\left([NaNO_2]_{LPS+Sample} - [NaNO_2]_{LPS-} \right)}{\left([NaNO_2]_{LPS+} - [NaNO_2]_{LPS-} \right)} \times 100$$

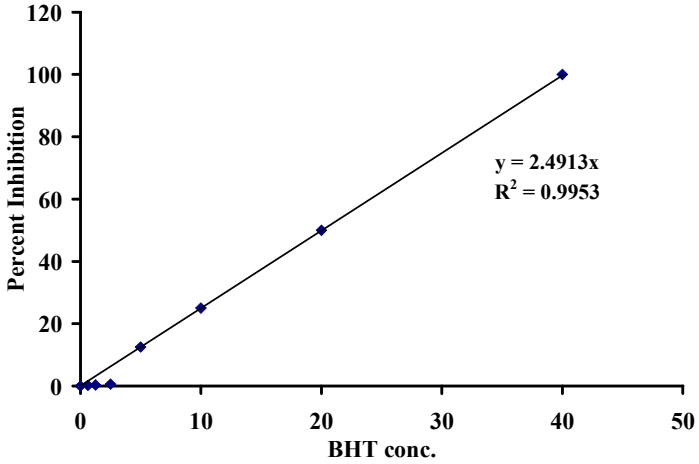
10. IC_{50} is obtained by regression analysis of dose-response curve plotting between percentage inhibition and sample concentration (Figure 34).



$$[NaNO_{\square}] = \frac{OD_{sample}}{slope}$$

$$[NaNO_{\square}] = \frac{OD_{sample}}{\square[\square\square}$$

Figure 33. The sodium nitrite standard curve to calculate nitrite concentration of samples



$$IC_{\square\square} = \frac{\square\square}{slope}$$

$$IC_{\square\square} = \frac{\square\square}{\square\square\square\square\square}$$

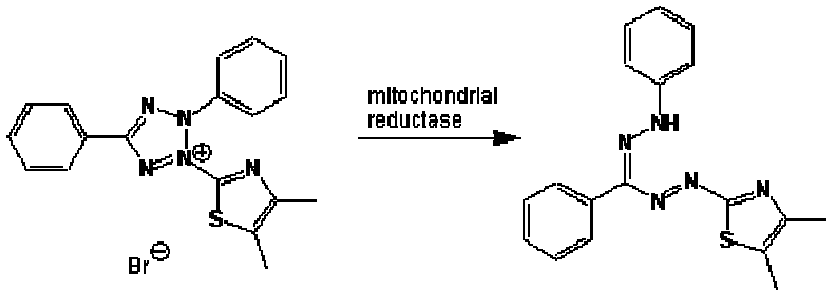
$$IC_{\square\square} = \square\square\square\square$$

Figure 34. The linear regression analysis to calculate IC_{50}

C-2). Mitochondrial function and cell viability using MTT assay

1). Principle

MTT is a standard colorimetric assay for determining the activity of mitochondria respiration. This assay could refer to cell growth, viability and cytotoxicity. A yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is reduced to purple water-insoluble formazan by mitochondrial succinate-tetrazolium reductase (EC 1.3.99.1) and in minor role by electron transport chain reaction and cytoplasmic reduction involving the pyridine nucleotide cofactors NADH and NADPH (see equation below). Some enzyme substrate could be reduced to water soluble formazan salt such as XTT, MTS and WST-1.



2). Reagents

1. 10× MTT stock solution
2. Dimethyl sulfoxide (DMSO)

3). Equipment

1. Micro-plate reader BIO-RAD model 550 using wavelength:
   - At 570 nm filter as measuring wavelength
   - At 630 nm filter as reference wavelength.

4). MTT Measurement

1. Allow the MTT solution to equilibrate at room temperature (15-30 min).

2. Prepare 10 ml (for 96-well plate) of 0.5 mg/ml MTT working solution by diluting MTT stock solution 1: 10 in complete DMEM.

3. Remove old medium in each wells and wash twice times with 100 μl PBS.

4. Add 100 μl of MTT working solution to each well containing cells.

5. Incubate 30 min at 37 °C.

6. Remove medium and wash twice with 100 μl PBS.

7. Add 200 μl DMSO and allow plate at room temperature for 30 min to dissolve dark blue crystal.

8. Mix thoroughly and Measure absorbance in micro-plate reader with a test wavelength 570 nm filter and a reference wavelength 630 nm within 30 min.

9. Convert absorbance to %cell viability using equation below:

$$\square\ cell\ viability = \frac{\left(OD_{sample}\right) \times \square\square\square}{\left(OD_{control}\right)}$$

C-3). Total RNA extraction using TRIzol reagent (Invitrogen)

1). Principle

TRIzol reagent (U.S. Patent No. 5,346,994) is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). During sample lysis, TRIzol reagent maintains the integrity of RNA, while disrupting cell and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the inter-phase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase. Co-purification of the DNA may be useful for normalizing RNA yields from sample to sample.

2). Reagents

1. TRIzol reagent (Invitrogen)
2. Chloroform (Merk)
3. Isopropyl alcohol (Merk)
4. 75% Ethanol in DEPC-treated water
5. DEPC-treated water (0.01% v/v diethylpyrocarbonate over night and autoclave)

3). Equipments

1. Refrigerated Centrifuge (Sorvall RC 50 plus)
2. Spectrophotometer (Shimadzu UV-160A) using wave length at 260 and 280 nm

4). Total RNA extraction

4.1). Homogenization

1. RAW 264.7 cells (2×10^6 cells/well in a 6-well plate) are washed twice with 1× cold PBS.

2. Lyse cells directly by adding 1 ml of TRIzol reagent to each wells of 6-well plate, and passing the cell lysates several times through a pipette.

4.2). Phase separation

1. Incubate the homogenized samples for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.

2. Add 0.2 ml of chloroform per 1 ml TRIzol reagent.

3. Cape sample tube securely. Shake tubes vigorously by hand for 15 sec and incubate them at room temperatures for 5 min.

4. Centrifuge the samples 12,000 ×g (10,000 rpm) for 15 min at 2 to 8 °C.

5. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an inter-phase, and a colorless upper aqueous phase.

6. RNA remains exclusively in the aqueous phase. The volume of aqueous phase is about 60% of the volume of TRIzol reagent.

4.3). RNA precipitation

1. Transfer the aqueous phase to a fresh tube , and save the organic phase if isolation of DNA or protein is desired.

2. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol.

3. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent used for the initial homogenization.

4. Incubate sample at -70 °C for over night and centrifuge at 12,000 ×g (10,000 rpm) for 10 min at 2 to 8 °C.

5. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4.4). RNA wash

1. Remove the supernatant.
2. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml TRIzol reagent used for the initial homogenization.
3. Mix the sample by vortexing and centrifuge at 7,500 ×g (6,500 rpm) for 5 min at 2 to 8 °C.

4.5). Re-dissolving the RNA

1. At the end of the procedure, briefly dry the RNA pellet (air-dry under laminar flow hood) for 15 to 30 min. It is important not to let the RNA dry completely as this will greatly decrease its solubility. Partially dissolved RNA sample have an $A_{260/280}$ ratio < 1.6.
2. Dissolve RNA in DEPC-treated water by passing the solution 2 to 3 times through a pipette tip, and incubating for 10 min at 55 °C.

4.6). RNA qualification and quantification

1. Total RNA concentration (ng/μl) is calculated from an equation:

$$RNA\ conc = A \times$$

2. The quality of RNA is determined using A_{260}/A_{280} ratio.

   A_{260}/A_{280} ratio < 1.65 mean:

   - Partially dissolved RNA sample
   - Sample homogenized in a small volume of TRIzol reagent
   - The aqueous phase is contaminated with the phenol phase
   - Incomplete dissolution of final RNA pellet

C-4). Reveres transcription using M-MLV reveres transcriptase (Invitrogen)

1). Principle

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) uses single-stranded RNA or DNA in the presence of a primer to synthesize a complementary DNA strand (cDNA). This enzyme is isolated from *E. coli* expressing a portion of the *pol* gene of M-MLV on plasmid. The enzyme is used to synthesize first-strand cDNA up to 7 kb.

2). Reagents

1. M-MLV RT reagent (Invitrogen)
   - 200 unit/μl M-MLV Reverse Transcriptase (Invitrogen)
   - 5× First-Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)
   - 0.1 M DTT
2. 40 unit/μl RNaseOUT Recombinant Riboneuclease Inhibitor (Invitrogen)
3. 500 μg/ml Oligo $(dT)_{16}$ (Operon)
4. 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH)

3). Equipment

1. Thermal Cycler (My Cycler, BIO-RAD)

4). First-Strand cDNA synthesis using M-MLV RT

1. A 20-μl reaction volume can be used for 1 ng-5 μg of total RNA or 1-500 ng of mRNA. Add the following components to a nuclease-free microcentrifuge tube:

Total RNA	2.0 μg
500 μg/ml Oligo $(dT)_{16}$	0.5 μl
10 mM dNTP mix	0.5 μl
DEPC-treated water	Add to total volume 13.0 μl

2. Heat mixture to 65 °C for 5 min and quick chill on ice. Collect the contents of tube by brief centrifugation and add:

5× First-Strand buffer	4.0 μl
0.1 M DTT	1.0 μl
RNaseOUT	0.5 μl

3. Mix contents of tube gently and incubate at 37 °C for 2 min
4. Add 0.5 μl of 200 unit/μl M-MLV RT and mix by Pipetting gently up and down
5. Start reaction using thermal cycler: 37 °C for 50 min and 70 °C for 15 min.

C-5). Polymerase Chain Reaction using *Taq* polymerase (Invitrogen)

1). Principle

Taq DNA polymerase is purified from *E. coli* expressing a cloned *Thermus aquaticus* DNA polymerase gene. This enzyme has both a 5'→ 3' DNA polymerase and 5'→ 3' exonuclease activity but lack a 3'→ 5' exonuclease activity. The enzyme consists of a single peptide with a molecular weight of approximately 94 kDa. *Taq* DNA polymerase is heat stable and will synthesize DNA at elevated temperatures from single-stranded templates in the presence of a specific primer.

2). Reagents

1. *Taq* PCR kit (Invitrogen)
   - 10× PCR buffer minus Mg (200 mM Tris-HCl pH 8.4 and 500 mM KCl)
   - 5 unit/μl *Taq* DNA Polymerase
   - 50 mM $MgCl_2$
2. 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH)
3. 10 mM specific primer (Appendix B-8)
4. Sterile deionized water (DI)

3). Equipment

1. Thermal Cycler (My Cycler, BIO-RAD)

4). Polymerase Chain Reaction

1. Add the following components to a PCR tube for 1 reaction master mix:

DI water	9.17 µl
10× PCR buffer minus Mg	1.50 µl
50 mM $MgCl_2$	0.45 µl
10 mM dNTP mixture	0.30 µl
10 mM specific F-primer	0.75 µl
10 mM specific R-primer	0.75 µl
5 unit/µl *Taq* DNA Polymerase	0.08 µl
Total volume	**13.00 µl**

2. 10% of cDNA (2 µl) from Reverse transcription (Appendix C-4) is used as the template and added in 13 µl master reaction mixture.

3. Mix contents of tube and centrifuge briefly to collect the contents to the bottom.

4. Incubate the tubes in a thermal cycler:

Denaturation (1 cycle)
- 94 °C 3 min

PCR amplification (35 cycles)
- 94 °C 45 sec
- 45 °C 30 sec
- 72 °C 60 sec

Final extension (1 cycle)
- 72 °C 10 min

5. Maintain reaction at 4 °C. The samples can be stored at 20 °C until use.

6. Analyze the PCR products by agarose gel electrophoresis and visualize by ethidium bromide staining.

C-6). Agarose gel electrophoresis

1). Principle

Electrophoresis is the study of the movement of charged molecules in an electric field. The generally used support medium is cellulose or thin gels made up of either polyacrylamide or agarose. Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA or PCR product. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field. Shorter molecules move faster and migrate farther than longer ones. The DNA is visualized in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. The advantages are that the gel is easily poured, does not denature the samples. The samples can also be recovered. The disadvantages are that gels can melt during electrophoresis, the buffer can become exhausted, and different forms of genetic material may run in unpredictable forms. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

2). Chemicals and Reagents

1. Agarose
2. 0.5× TBE
3. 10 mg/ml ethidium bromide
4. 6× Loading dye
5. 500 μg/ml 100 bp DNA ladder (New England BioLabs Inc.)

3). Equipments

1. Gel casting tray and Comb
2. Electrophoresis chamber and Power supply
3. Automated gel doc system (Bioimagine System, Syngene, USA)

4). Procedure

1. For a 1.8% agarose gel, weigh out 0.72 g of agarose into a flask and add 40 ml of 0.5× TBE for 5×6 cm^2 gel casting tray. Or weigh out 1.44 g of agarose into a flask and add 80 ml of 0.5× TBE for 10.5×6 cm^2 gel casting tray.

2. Heat solution in a microwave until agarose is completely dissolved (approximately 30-45 sec).

3. Allow to cool in a water bath set at 50-55 °C for 10 min.

4. Prepare gel casting tray by sealing ends of gel chamber with appropriate casting system. Place appropriate number of combs in gel casting tray.

5. Add 10 μl (for 40 ml gel) or 20 μl (for 80 ml gel) of 10 mg/ml ethidium bromide to cooled gel and pour into gel casting tray. Allow to cool for 15-30 min at room temperature. Gels can also be placed in a cold space and used the following day.

6. Remove comb(s), place in electrophoresis chamber and cover with 0.5× TBE buffer.

7. Add 6× loading dye to samples or 0.5 μg 100 bp Ladder. As a guideline, add 1 μl of 6× Loading dye to a 5 μl PCR/DNA solution.

8. Load DNA and 100 bp Ladder onto gel. The standard pattern is shown in Figure 35.

9. Start electrophoresis at 100V for 45-60 min.

10. Visualize DNA bands using automated gel doc system.

11. Calculate band intensity using SiconImage program.

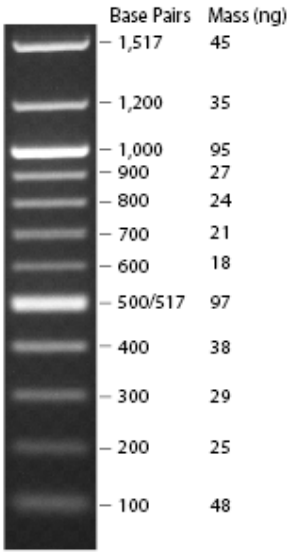


Figure 35. The 100-bp DNA ladder from New England BioLabs Inc.

The 100-bp DNA Ladder visualized by ethidium bromide staining on a 1.3% TAE agarose gel. Mass values are for 0.5 μg/lane.

C-7). Transfection of RAW 264.7 cells with pNFκB-Luc using FuGENE6

1). Principle

Stratagene's Path Detect pNFκB-Luc is designed for the *in vivo* activation of NF-κB signal transduction pathway. In Figure 36, the inducible pNFκB-Luc reporter plasmid contains the luciferase reporter gene driven by a basic promoter element (TATA box) plus 5× NF-κB *Cis*-enhancer elements ($[TGGGGACTTTCCGC]_5$). This plasmid is particularly suited for the *in vivo* readout of signal transduction pathway since this pathway is convergent point of NF-κB signaling pathway.

2). Reagents

1. 1 μg/μl pNFκB-Luc (Stratagene)
2. FuGENE6 Transfection reagent (Roche)
3. Serum free DMEM
4. Complete DMEM

3). Procedure

1. One day before the transfection experiment, RAW 264.7 cells (1×10^5 cells/well) were seeded in a 48-well plate and allowed to adhere for 24 hrs (30% confluence).
2. Prepare the transfection solution for 1 reaction as follow:
   - Pipette 49.5 μl serum free DMEM into a micro centrifuge tube
   - Pipette 0.3 μl the FuGENE6 Transfection reagent directly into the medium without allowing contact with the walls of plastic tube
   - Vortex for one second or flick the tube to mix. Incubate for 5 min at room temperature.
3. Add 0.2 μg of pNFκB-Luc plasmid (0.2 μl)
4. Tap the tube or vortex for one second to mix the contents. Incubate the transfection reagent-plasmid complex for 20 min at room temperature.
5. Add 150 μl complete DMEM to make a complete transfection medium.

6. Remove old medium and replace a complete transfection medium in a drop-wise manner. Swirl the wells to ensure distribution over the entire plate surface.

7. Return cell to the incubator for 24-hours incubation period and then start the experiment.

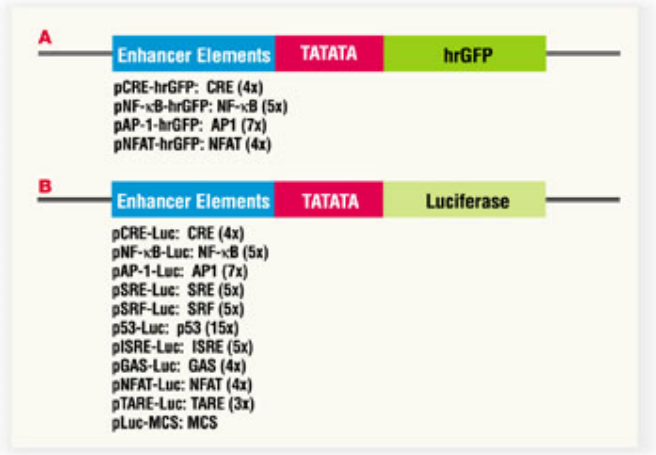


Figure 36. Structure of Path Detect *Cis*-reporting system plasmids using vitality hrGFP or luciferase

Each *Cis*-reporter plasmid contains tandem repeats of one of the indicated enhancer elements; the multiple cloning site of pLuc-MCS contains standard restriction sites for insertion of any enhancer. For each reporter plasmid, the enhancer element and TATA box control expression of the reporter gene (**A**: Vitality hrGFP or **B**: luciferase). The hrGFP gene is controlled by one of four different enhancer elements. The luciferase gene is controlled by one of 10 enhancer elements.

C-8). Luciferase activity

1). Principle

American firefly (*Photinus pyralis*) luciferase catalyzes the following chemiluminescent oxidation-reduction reaction:

$$Luciferin + ATP + O_{\square} \rightarrow Oxyluciferin + AMP + PPi + CO_{\square} + light(\square\square\square nm)$$

The luciferase assay is highly sensitive. The background activity is low because mammalian cell do not contain endogenous luciferase. luciferase in the cell lysates catalyzes the chemiluminescent reaction, which emits light that remain nearly constant for several min.

2). Reagents

1. Luciferase assay kit (Stratagene)
   - Luciferase assay buffer
   - Luciferase assay substrate
   - Luciferase cell lysis buffer
2. 1× PBS

3). Equipments

1. Refrigerated Centrifuge (Sorvall RC 50 plus)
2. Tropix TR 717 Luminometer Plate Reader (Applied Biosystems, Foster City, CA)

4). Cell lysis Procedure

1. Being careful not to dislodge any of the cells, remove the media from the tissue culture plate wells and wash the cells twice with 1× PBS.

2. Using a Pasteur pipette, remove as much PBS as possible from each well.

3. Make 1× cell lysis buffer by adding 4 ml of distilled water per 1 ml of the 5× cell lysis buffer. Equilibrate the lysis buffer to room temperature before use.

4. Cover the cells by adding approximately 200 μl of 1× cell lysis buffer to each well.

5. Incubate the plate at room temperature for 15 min, swirling occasionally.

6. Scrape the cells and buffer from each well into separate microcentrifuge tubes. Place the tubes on ice.

7. Vortex the microcentrifuge tubes for 15 sec. Spin the tubes in a microcentrifuge at 12,000 ×g for 2 min at 4 °C.

8. Transfer the supernatant from each tube to a new microcentrifuge tube.

9. Immediately assay the supernatant for luciferase activity according to the protocol in performing the luciferase Activity Assay or store the supernatant at −80 °C for later use.

5). Luciferase activity procedure

1. Prepare the luciferase substrate-assay buffer mixture by adding all of the assay buffer (10 ml) to the vial containing the lyophilized luciferase substrate and mixing well.

2. Divide the luciferase substrate-assay buffer mixture into aliquots of an appropriate size to avoid multiple freeze-thaw cycles.

3. Allow the luciferase substrate-assay buffer mixture to reach room temperature. Allow the supernatant from step 9 in Cell lysis procedure to reach room temperature.

4. Add 100 μl of the luciferase substrate-assay buffer mixture to a 96-well white plate.

5. Add 20 μl of supernatant to a 96-well white plate, mix, gently and immediately put into the luminometer.

6. Begin measuring the light (RLU) produced from the reaction ~8 sec after adding the supernatant using an integration time of 30 sec (use the same delay time for all of the samples).

7. Data are expressed in the Relative luciferase Activity (RLA) as follow:

$$RLA = \frac{RLU_{sample}}{RLU_{spon\,\square\square ious}}$$

C-9). Total protein extraction using RIPA lysis buffer

1). Principle

RIPA (Radio-Immunoprecipitation Assay) buffer enables rapid, efficient cell lysis and solubilization of protein from both adherent and suspension cultured mammalian cells. It has long been a widely used lysis and wash buffer for small-scale affinity pull-down applications, such as immunoprecipitation, since most antibodies and protein antigens are not adversely affected by the components of this buffer. In addition, RIPA buffer minimizes non-specific protein-binding interactions to keep background low, while allowing most specific interaction to occur, enabling studies of relevant protein-protein interactions.

2). Reagents

1. RIPA lysis buffer (Santacruz Biotechnology, Inc.)
2. 1× TBS, 1% Nonidet P-40, 0.5% Sodium deoxychorate, 0.1% SDS, 0.004% Sodium azide, 1% PMSF, 1% Sodium orthovanadate and 1% protease inhibitor cocktails.
3. 1× PBS

3). Equipment

1. Refrigerated Centrifuge (Sorvall RC 50 plus)

4). Procedure

1. Remove culture medium and rinse cell twice with 1× cold PBS
2. Harvest cell using cell scraper in to microcentrifuge tube and centrifuge for 5 min at 10000 rpm.
3. Add 50 μl RIPA buffer, gently mix using pipette up and down, and shake for 30 min at 4 °C.

4. Centrifuge cell lysates at 12000 rpm for 10 min at 4 °C. The supernatant fluid is the total cell lysates. Transfer the supernatant to a new microcentrifuge tube and store at −70 °C for a month.

C-10). Measurements of total protein concentration using Bradford method

1). Principle

The Bio-Rad assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shift from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to the primarily basic and aromatic amino acid residues, especially arginine. The extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

2). Reagents

1. 5× Bio-Rad protein assay reagent
2. 1 mg/ml Standard Bovine Serum albumin (BSA)

3). Equipment

1. Spectrophotometer (Shimadzu UV-160A) using wavelength at 595 nm

4). Microassay procedure

1. Prepare five dilution of BSA standard which is representative of protein solution to be tested. The linear range of assay is 1.2 to 10 μg/ml.
2. Pipette 800 μl of standard and sample solution into a clean, dry test tube. Protein solutions are assay in triplicate.
3. Add 200 μl of dye reagent concentrate to each tube and vortex.
4. Incubate at room temperature for 5 min. Absorbance will increase over times; sample should be incubated at room temperature for no more than 1 hrs.
5. Measure absorbance at 595 nm.
6. Determine protein concentration by using protein standard curve.

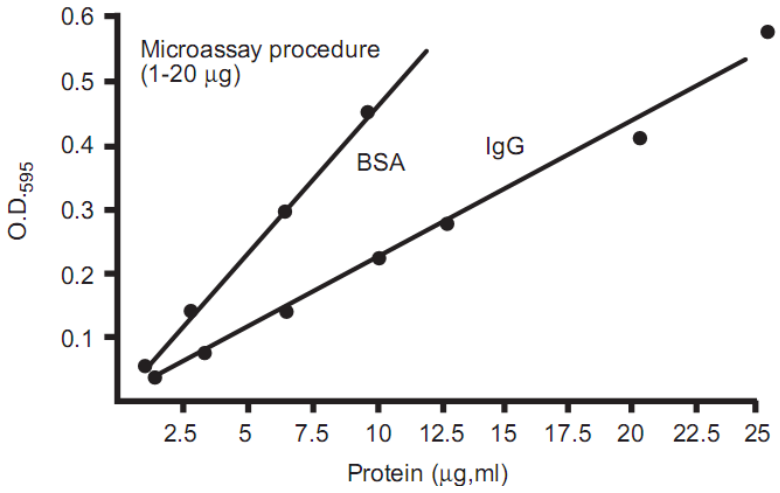


Figure 37. Protein standard curve to calculate total protein concentration of samples

C-11). SDS-polyacrylamide gels electrophoresis and Western blot

1). Principle

Electrophoresis is the study of the movement of charged molecules in an electric field. The generally used support medium is cellulose or thin gels made up of either polyacrylamide or agarose. Polyacrylamide gel is widely used for larger molecules like proteins. Polyacrylamide gels are prepared by the free radical polymerization of acrylamide and the cross linking agent *N N*' methylene bis acrylamide as following:

$$Acrylamide + NN\text{'}methylene\,bis\,acrylamide \xrightarrow{APS+TEMED} Polyacrylamide$$

The molecular weight of proteins is estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS) and a reducing agent 2-β-mercaptoethanol. SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules while mercaptoethanol assists the protein denaturation by reducing all disulfide bonds.

2). Chemicals and Reagents

1. 40% Bis/Acrylamide (Amresco)
2. 1.5 M Tris, pH 8.8
3. 1.0 M Tris, pH 6.8
4. 10% SDS
5. 10% APS
6. *N, N, N', N'*-tetra methylethylenediamine or TEMED (Sigma)
7. 1× Running buffer
8. 4× Protein-loading dye
9. 70% alcohol
10. Sterile distilled water
11. 7-175 kDa Protein marker (New England Biolabs, Inc.)
12. 1× transfer buffer

13. Picowest detection reagent

14. Striping buffer

3). Equipments

1. Glass plate, casting stand, combs
2. Vertical gel-running set, tank and power supply
3. Water bath
4. Mini Trans-Blot Electrophoretic Transfer cell
5. Transfer set, tank and power supply

4). Gel preparation procedure

1. Clean glass plats with 70% alcohol.
2. Assemble glass plates in casting stand.
3. Prepare separating and stacking mixture (Table 19). APS and TEMED are added in the last step before adding mixture into gel.
4. Add mixture of separating gel and over-layer with sterile distilled water to aid edge polymerization and remove air bubbles. Incubate to polymerize for 90 min.
5. Pour off over-layered water and dry with 3M-filter paper.
6. Add stacking mixture to gel until flash with top edge of small glass plate.
7. Add comb, make sure it's centered. Allow stacking gel to polymerize for 30 min.
8. Remove comb and wash wells to remove bubbles. Make sure well are clean with no residual acrylamide.
9. Assemble glass plate gels in gel-running set and fill with 1× Running buffer inside electrode assembly and outside.

Table 19. The content of mixture to prepare polyacrylamide gel

Reagents	Separating gel (12% Acrylamide)	Stacking gel (4% Acrylamide)
Sterile distilled water	580×4 μl	752×3 μl
40% Bis/Acrylamide	750×2 μl	300 μl
1.5 M Tris, pH 8.8	540×2 μl	-
1.0 M Tris, pH 6.8	-	375 μl
10% SDS	50 μl	30 μl
10% APS	50 μl	30 μl
TEMED	2 μl	2 μl
Total volume	**5 ml**	**3 ml**

5). Sample preparation and gel electrophoresis

1. Mix 100 μg whole cell lysates and 4× Protein-loading dye with ration 3 μl: 1 μl loading dye. The total volume is no more than 25 μl.

2. Prepare 6 μl standard protein marker in 4× Protein-loading dye using the equal volume with used in sample.

3. Boil the mixture for 5 min at 100 °C in water bath. Allow immediately on ice. Unused samples may be stored at -20 °C.

4. Load standard protein marker and samples into gel.

5. Attach to electrode assembly/tank to power supply. Run at 80 V for 30 min until samples and standard move to separating gel.

6. Increase voltage to 150 V for 90 min (dye and protein marker lower than 30 kDa are run off).

7. Gel is removed to either strain with Coomassie blue or transfer to nitrocellulose for Western blot.

6). Transfer to nitrocellulose membrane

1. Place gel in dish containing 1× transfer buffer. Allow to soak for 10 min.

2. Per gel, cut to size: 1 piece of nitrocellulose and 4 pieces of 3M filter paper. Place all in 1× transfer buffer.

3. Layer onto Mini Trans-Blot Electrophoretic Transfer cell from bottom to top (black plate to white plate)

a. 1 piece Transferring sponge

b. 2 pieces 3M filter paper

c. polyacrylamide gel

d. nitrocellulose or PVDF membrane

e. 2 pieces 3M filter paper

f. 1 piece Transferring sponge

4. Make sure there are no air bubbles.

5. Set up at a constant current at 35V for overnight and finalize at 100V for 30 min.

6. Stain nitrocellulose with Ponceau S and gel with Coomassie blue to ensure protein transfer. Rinse with running distilled water to remove Ponceau S. Keep blot moist for storage.

7). Immunoblot

1. Block non-specific binding sites on nitrocellulose membrane using 5% non-fat dried milk in 1× TTBS for 1 hrs at room temperature on a orbital shaker.

2. Wash the membrane for 2×5 min with fresh changed of 1% non-fat dried milk in 1× TTBS at room temperature.

3. Dilute primary antibody in 5% non-fat dried milk in 1× TTBS. The dilution factor of each antibody is shown in Table 20.

4. Incubate the membrane in diluted primary antibody overnight at 4 °C on an orbital shaker.

5. Wash the membrane for 3×5 min with fresh changed of 1% non-fat dried milk in 1× TTBS at room temperature.

6. Dilute HRP-labeled secondary antibody in 5% non-fat dried milk in 1× TTBS. The dilution factor of each antibody is shown in Table 20.

7. Incubate the membrane in diluted secondary antibody for 1 hrs at room temperature.

8. Wash the membrane for 3×10 min with fresh changed of 1× TTBS at room temperature.

9. The membrane is ready to detect specific band.

8). Detection with chemiluminescence reagent

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1. Mix an equal volume of reagent 1 and reagent 2 allowing sufficient total volume to cover the membranes.

2. Drain the access wash buffer from the washed membrane and place them, protein side up on the suitable clean surface. Pipette the mixed detection reagent on the membranes.

3. Incubate 1 min at room temperature.

4. Drain off access detection reagent by holding the membrane gently with forceps and touching the edge against a tissue.

5. Place the blot protein side up on the X-ray film cassette and cover with plastic wrap. Gently smooth out any air bubbles.

6. Expose to autoradiography film and develop film.

9). Stripping and Re-probing

1. Submerge the membrane in stripping buffer and incubate at 50 °C for 30 min with occasional agitation.

2. Wash the membrane for 2×10 min with fresh changed of 1× TTBS in the large volume at room temperature.

3. Repeat immunoblot protocol with a appropriate primary and secondary antibodies.

Table 20. The dilution of antibodies use in Immunoblot

Primary antibodies (Ratio)	Secondary antibodies (Ratio)
- Mouse monoclonal anti-human p-IκBα Ab (1:200) - Mouse monoclonal anti-human IκBα Ab (1:200) - Mouse monoclonal anti-human p-ERK1/2 Ab (1:2000)	Anti-mouse IgG HRP-linked Ab (1:2000)
- Rabbit polyclonal anti-human ERK1 Ab (1:500) - Rabbit polyclonal anti-human ERK2 Ab (1:500) - Rabbit polyclonal anti-human Actin Ab (1:2000)	Anti-rabbit IgG HRP-linked Ab (1:2000)

VITAE

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List of Publication and Proceeding

Hiransai, P., Ratanachaiyavong, S., Itharat, A., Graidist, P., Ruengrairatanaroj, P. and Purintrapiban, J. 2010. Dioscorealide B Suppresses LPS-Induced Nitric Oxide Production and Inflammatory Cytokine Expression in RAW 264.7 Macrophages: The Inhibition of NF-κB and ERK1/2 Activation. *J Cell Biochem*. 109:1057-63.

Hiransai, P., Itharat, A. and Ratanachaiyavong, S. 2008. Dioscorealide B inhibited LPS-induced nitric oxide production and pro-inflammatory cytokine expression. *Naresuan Phayao J.* 1 supplement (1): 194 (Abstract from the 2nd International Conference on Natural Products for Health and Beauty, 17th-19th December 2008, Payao, Thailand)

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the King and King Mongkut′s Institute of Technology Ladkrabang (Eds), Abstract: the 34th congress on Science and Technology Thailand (STT34), the Science Society of Thailand under the Patronage of Hismajesty the King, Thailand, p. 226.