CHAPTER 1

Introduction

1.1 Principle, Theory, Rationale, and/or Hypothesis

Nowadays, Inflammation is recognized as an overwhelming concern to the health status of population and the underlying basis of significant pathological diseases. Inflammation is a biological reaction, in response to disrupted tissue homeostasis which could be either acute or chronic. Acute inflammation is a destruction process of tissues that could recruit leukocytes and blood derived products into the injured tissue. This migration of these molecules lead to vasodilation, increased vascular permeability, and increased blood flow in a local vasculature. Infection by microbial, injury or trauma (in the absence of microbial infection) and exposure to foreign particles/irritants/pollutants are also potent activators of inflammation. Thus, the main functions of inflammation are rapid degradation or elimination of the disturbance, removal of damaged tissue, and restoration of tissue homeostasis.

However, in prolonged or chronic inflammation probably plays an important role in chronic diseases, such as metabolic syndromes, diabetes mellitus type 2 (DM2), cancer, and rheumatoid arthritis. Mostly, the important markers of inflammation are elevated including inflammatory cytokines and mediators, such as nitric oxide (NO), interleukin that can induce cellular signaling pathways including MAPKs, PI3-K/Akt and NF-κB signaling pathways. From previous knowledge, drugs used for treatment of anti-inflammation or pathogenesis involving in inflammation have several side effects. Recently, the researchers therefore have tried to investigate the novel knowledge to treat or relieve the disorders derived from inflammation by phytochemicals from natural products that have no or less side effect.

Asian people have used natural products to prevent or treat some illnesses for decades. Several medicinal herbs provide health benefit, such as ginger and green tea

that showed anti-oxidative stress and anti-inflammation properties. *Anoectochilus roxburghii* and *Anoectochilus formosanus* are popular in China, Vietnam and Taiwan to consume as hot tea or decoction. Their diverse pharmacological effects have been reported with possibilities to use for the treatment of diabetes, liver cancer, and cardiovascular disease that are involved in inflammation.

Anoectochilus species could be found in Asian countries and has been used as folk medicine in several countries such as China, Taiwan and Vietnam. Kinsenoside isolated from *Anoectochilus roxburghii* and *Anoectochilus formosanus* exhibits anti-hyperglycemic, anti-oxidative stress, anti-inflammation, and hepatoprotective properties. This study is interested in biological effects of *Anoectochilus burmannicus* or Nok-Kum-Fai found in Northern Thailand. Therefore, the hypothesis of this study is *Anoectochilus burmannicus* extract might exert biological properties against oxidative stress, inflammation and inflammation-induced insulin resistance. The knowledge from this study will provide supportive evidence to develop this plant to be implemented as an alternative medicine or functional food in order to prevent and/or treat chronic diseases which have resulted from inflammation.

1.2 Literature review

Lifestyle and behavior are important for good or bad health. For example, overnutrition leads to overweight and obesity, which are major health problems worldwide (1). Recently, at least 2.8 million of adults die each year as a cause of being overweight or obese, and the rates of obesity in children have increased (1, 2). Recently, children intend to have chronic diseases involving in metabolic syndrome including insulin resistance and DM type 2, which are serious problems in Thailand and worldwide (3-5). From previous study, there is a linkage between obese metabolic syndromes and inflammation (6, 7). Several studies reported that during overconsumption, expression of pro-inflammatory cytokines or biomarkers of inflammation in adipose tissue and the innate immune cells is increased (7, 8). Moreover, overconsumption could increase the level of reactive oxygen species (ROS) that support incident of inflammation (9). Prolonged of this situation leads to imbalance homeostasis resulting in many chronic diseases. Not only metabolic syndromes.

Inflammation also contributes to other diseases including cancer (10-12) and rheumatoid arthritis (13-16).

1.2.1 Inflammation

Inflammation is the process by which the immune system response to harmful stimuli. The host cells attempt to remove the harmful stimuli such as necrotic cells then initiate the process of repair (17). The four important signs of inflammation are redness, heat, swelling and pain. As of the late 19th century, the impairment of function is a fifth sign of inflammation (18). Acute inflammation has beneficial for health by recovery damage cellular or tissue in a short time (19). However, sustained, excessive or inappropriate inflammation is the cause of numerous diseases including metabolic syndromes (1, 6, 20), neurodegenerative diseases (5, 18), cancer (10-12), and rheumatoid arthritis (13, 14).

1.2.2 The process of inflammation

Inflammation is a complex process (Figure 1.1). After exposure to the activator of inflammation, the vessels flow are changed due to the release of nitric oxide (NO) or other mediators resulting in vasodilation and increasing blood flow leading to heat and redness as a consequence. The slowing of blood flow can decrease blood pressure resulting in hyper viscosity of blood in stasis step. Later, the margination step leukocytes are accumulates and adhere at the epithelial cells of blood vessel at the site of injury. After the changing of vasculature, transudate (protein-poor filtrate of plasma) gives way to exudate (protein-rich filtrate) into extracellular tissues (21).

The second step is vascular leakage and edema, whereby vessel permeability changes are due to the activation of many mediators, such as histamines, bradykinins, leukotrienes that are produced by leucocytes (22). These mediators have an effect on endothelial cell contraction that result in wider intercellular gaps within the endothelial layer. Thus, leaking of protein-rich fluid (exudate) into the extracellular tissues that causes a reduction of intravascular osmotic pressure and increase in extravascular/interstitial osmotic pressure. The increasing of interstitial osmotic pressure leads to edema (water and ions).

Finally, emigration of leukocytes into extravascular tissue, leukocytes leave the vasculature through the margination and rolling, when fluids leave the vessel, leukocytes are margination along the endothelial surface. In the process of rolling, leukocytes tumble slowly along the endothelium, adhere though surface adhesive molecule on endothelial cell and their complementary ligands on leukocyte (23). Importantly, the leukocytes are activated when they adhere with selective ligands from the selectin family or adhesion molecule such as E-selectin was selective with endothelium, P-selectin was selective with endothelium and platelets, L-selectin was selective with leukocytes (24, 25). However, the selectin families are overexpressed on endothelium by cytokines (TNF-α, IL-6 or IL-1) at injury sites conjugated with leukocyte surface molecule (25). Moreover, rolling of leukocytes can activates ligands bind on chemokine receptors to generates integrins (LFA-1), leading to tightly adhesion of leukocytes. Then, leukocytes leave the microvasculature by the joint between endothelial cells (paracellular route) or directly through endothelial cells (26). Once inside tissues, leukocytes migrate to the injury site by chemoattractants. The effect of leukocytes in extravascular space releases chemical mediators which recruit other leukocytes to remove the stimulus. These occur by the distribution and synthesis of adhesion molecule on intra/extravascular and enhance a conjugation between adhesion molecules (27).

However, inflammation could not be last long, thus there are also have a prevent inflammation mechanisms. Several kinds of molecules as free radicals, nitrogen intermediates and prostaglandin E2 (PGE2) have a dual role in both promoting and suppressing inflammation. Sometime, it cannot regulate the duration of inflammation that leads to chronic inflammation (28, 29). Chronic inflammation can cause tissue damage and contribute to other chronic diseases such as rheumatoid arthritis, cancer and diabetes. Moreover, inflammatory cells, especially macrophages generate amount of growth factors, chemokines, cytokines, ROS and nitrogen species that could cause DNA damage leading to mutations (30, 31). Continuous of activated macrophages might lead to prolong of tissue damage and impairment of microenvironment induced cell proliferation, thus predisposes chronic inflammation to neoplasia.

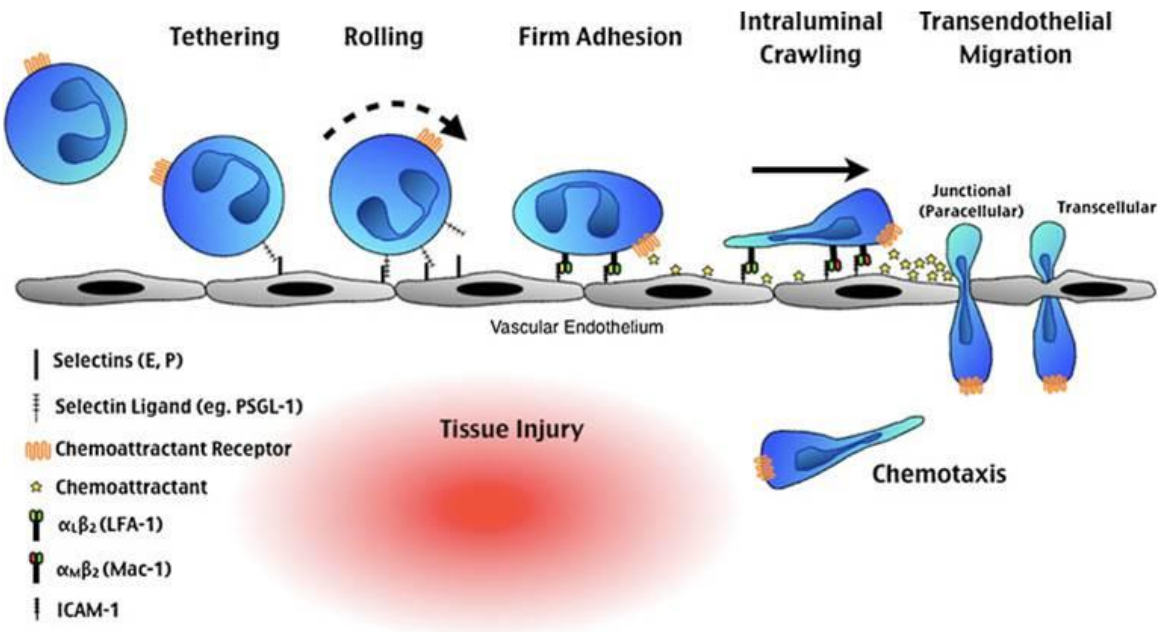

Figure 1.1 The process of inflammation: leukocytes rolling into an injury site (24).

1.2.3 Chemotaxis and chemical mediator in inflammation

Chemotaxis is the movement of leukocyte to injury sites by stimulation, also called chemoattractant or chemotic factor such as lipopolysaccharide or other mediators produced by bacterial membranes can recruit leukocytes into the infection site (32, 33). Activated leukocyte can activate oxidative reaction, arachidonic acid metabolism, increase the role of adhesion molecule on leukocytes and stimulate the secretion of enzymes in the lysosome (33).

Chemical mediator in inflammation could be released from plasma precursor in the blood or released from inflammatory cells. Mostly, mediators have a short half-life, some mediators can further activate cells to produce secondary mediators, thus extend the inflammatory reaction. The important mediators involved in inflammation including pro-inflammatory cytokines (TNF-α, IL-1, IL-6) (34), arachidonic acid (AA) metabolites (35-37), nitric oxide (38-40), and oxygen-derived free radicals (41-43).

1.2.3.1 Arachidonic acid (AA) metabolites

The mediators in AA metabolism are prostaglandins and leukotrienes, also called eicosanoids. AA is a polyunsaturated fatty acid that found in the phospholipid of cell membrane, especially in leukocyte (35, 37). In inflammation, tissue damage from injury or several stimulus can activate phospholipase A2 (PL-A2), which produced AA (44), and then AA can shift into many mediators (Figure 1.2) such as the followings

- Prostacyclin I2 (PGI2) and prostaglandin E2 (PGE2) : increases vascular permeability and vasodilation (37)
- Thromboxane A2 : vascular contraction and platelet aggregation (45, 46)
- Leukotriene C4 leukotriene D4 and leukotriene E4 : increase vascular permeability and vascular contraction (47, 48)
- Leukotriene B4 : chemotactic factor and stimulate leukocyte adhesion (49, 50)
- PGE2 : pain and fever (36)

Under pathological conditions, LPS is one of important contributors that stimulates the overproduction of arachidonic acid (AA) by activating phospholipase A2 and C (Figure 1.2).

The important mediator of AA is prostaglandins, which are catalyzed by cyclooxygenases (COX) enzymes (35, 36, 51). Cyclooxygenases (COXs) are enzymes that consist in the rate-limiting step that catalyze arachidonic acid to prostaglandin H2 (PGH2). PGH2 is a precursor to many kinds of prostaglandins including PGE2. The metabolites of PGH2 are an eicosanoids. Some eicosanoids such as PGE2 effect on nerve endings hypersensitive and others lead to inflammation. However, during inflammatory reactions, eicosanoids initiates an inflammation and mediates resolution.

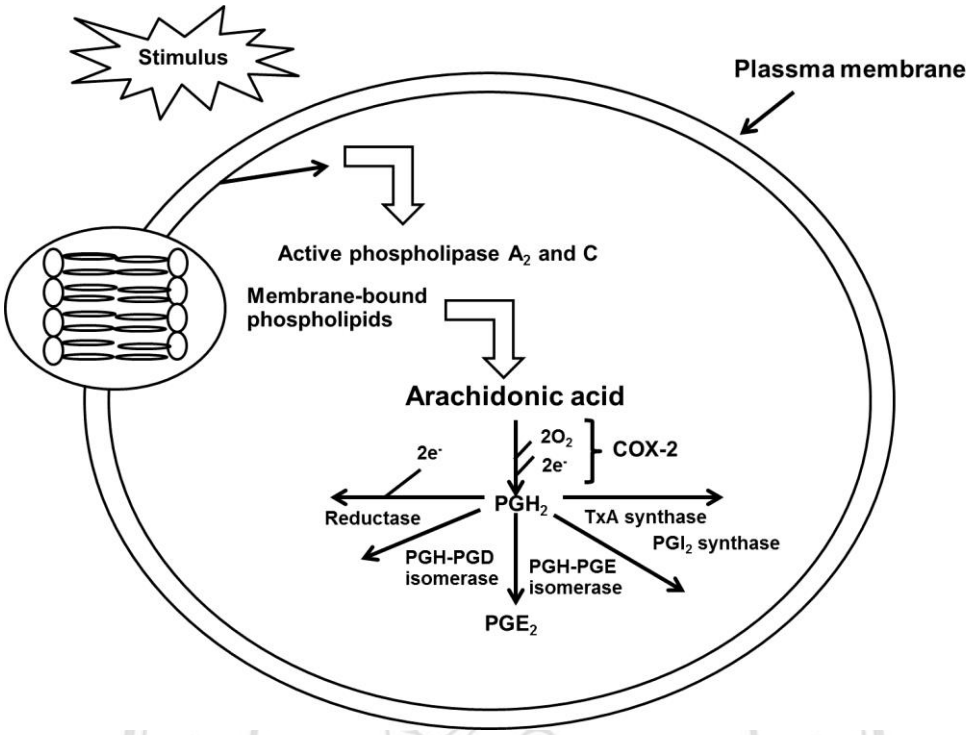

Figure 1.2 The biosynthesis pathway of the prostaglandins (51).

The COX enzyme family has two isoforms as COX-1 and COX-2 (52) as shown in Figure 1.3. COX-1 is a membrane-bound hemoglycoprotein that constitutively expressed in almost all tissues that synthesizes prostaglandin for homeostatic functions including platelet function and regulating renal blood flow (53). In contrast, COX-2 is an inducible COX isoform which consists in some tissue including brain and kidney. COX-2 expression is highly constrained, in contrast during inflammation COX-2 is excessively upregulated (54). LPS and pro-inflammatory cytokines such as IL-1 and TNF-α could stimulate COX-2 synthesis in many cell types, such as endothelial cells, chondrocytes, and macrophages (55).

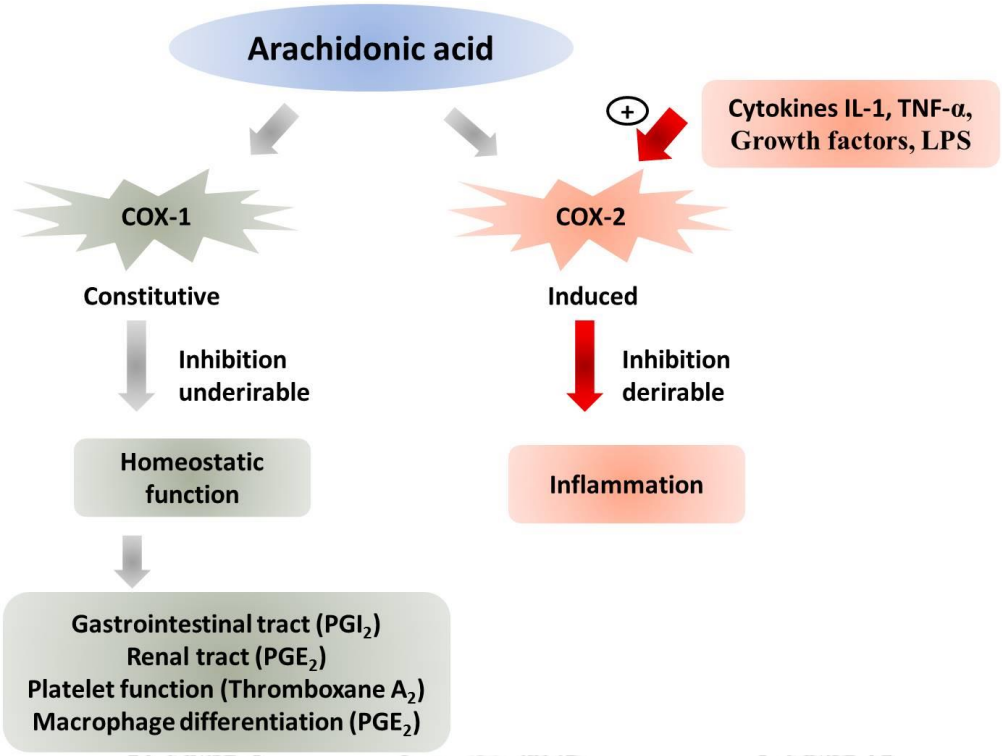

Figure 1.3 The current COX concept (53).

In the inflammation process, peroxynitrite, which is the coupling product of nitric oxide (NO) and superoxide anion, and COX-2 are especially important in inflamed cells. This is because of inducible NO synthase (iNOS) and COX-2 are "immediate-early" gene that are hightly expressed when expose to inflammatory stimuli such as LPS. Therefore, COX-2, is the first important target for anti-inflammation therapy due to COX-2 affect a broad range of mechanisms implicated in the process of many processes such as carcinogenesis, including angiogenesis, apoptosis, and immune function (51).

1.2.3.2 Cytokines

Cytokines are small polypeptides released by cells, in particular lymphocyte and macrophage, which have been activated and have a specific effect on the interactions and communications between cells (56). Releasing cytokines affect the behavior of other cells, and sometimes the releasing cells themselves (57). Cytokine is also called as lymphokine, monokine, chemokine, or interleukin (58). They play several roles as autocrine action, which act on secreted cells, paracrine action, which act on nearby cells, or endocrine action, which act in some distant

cells (34). They could be even pro-inflammatory cytokines or anti-inflammatory cytokines. Several studies showed cytokines/chemokines are involved in not only the initiate inflammation but also the resistance of pathogenesis, such as autoimmune, infectious, and neoplastic diseases (34).

1.2.3.2.1 Pro-inflammatory cytokines

Activated macrophage or inflamed cells generate pro-inflammatory cytokines during inflammation reaction (59, 60). There are abundant evidences showing that impotent cytokines, such as IL-1β, IL-6 and TNF-α, involve in the up-regulation of the inflammatory reaction (60).

IL-1β is released by immune cell such as monocytes, microglia, mast cells and macrophages as well as and other cells including fibroblasts, endothelial and neuronal (61). IL-1β is important for regulation of homeostatic functions in the normal organism, such as controlling of feeding, sleep, temperature and during cell injury, and inflammation (62, 63). In patients with various infections, inflammation, trauma (surgery) and autoimmune disorders, IL-1β production is highly excessive (64). IL-1β induces the production of substance P and PGE2 in neuronal and glial cells that lead to inflammation (63). Moreover, IL-1β is related to inflammation-induced insulin resistance and impairment of organ function in DM type II. From previous study of Jager (2010), IL-1β acted on insulin signaling by suppressing IRS-1 expression through ERK pathway (65).

IL-6 is a multifunctional cytokine that have a protective effect by suppressing the level of other pro-inflammatory cytokines during acute responses (66, 67). For example, IL-6 can stimulate the production of IL-1 receptor antagonist (68, 69), which is an anti-inflammatory mediator. However, IL-6 also plays roles on chronic inflammation driving a pathogenesis(67, 70-72).

The recruitment of monocytes by inducible IL-6 to the area of inflammation is the key to switch acute to chronic inflammation (Figure

1.4). During acute inflammation, host defense system can activates endothelium cells to produce IL-6 that can turn to activate an endothelium cells to release more IL-6 Besides, releasing IL-6 could stimulate a recruitment of neutrophils. In addition, IL-6 is binding on sIL-6R, secondly the complex of IL6/sIL6R can bind with gp130 receptor to induce trans-signaling through gp130 leads to monocyte recruitment. Achievement of this situation too long leads to increasing of MCP-1 secretion. MCP-1 is a chemokine, leading to a chemokine shift favoring monocyte recruitment. Moreover, IL-6 can induce neutrophilic apoptosis by polymorphonuclear neutrophils (PMN), in macrophage. After decreasing of neutrophils in inflammatory site and blood monocytes whereas accumulation of macrophages will increase and then it would be differentiate into inflammatory cells, which complete phagocytosis and destruction of the injurious agents. Several days, the monocytes and macrophage in the lymph node are differentiate into and dendritic cells, leading to upregulation of HLA class II antigen membrane expression and recruit co-stimulatory molecules such as CD80 and CD86. These cells are contributing to generate an immune response and induce chronic inflammation (70, 73).

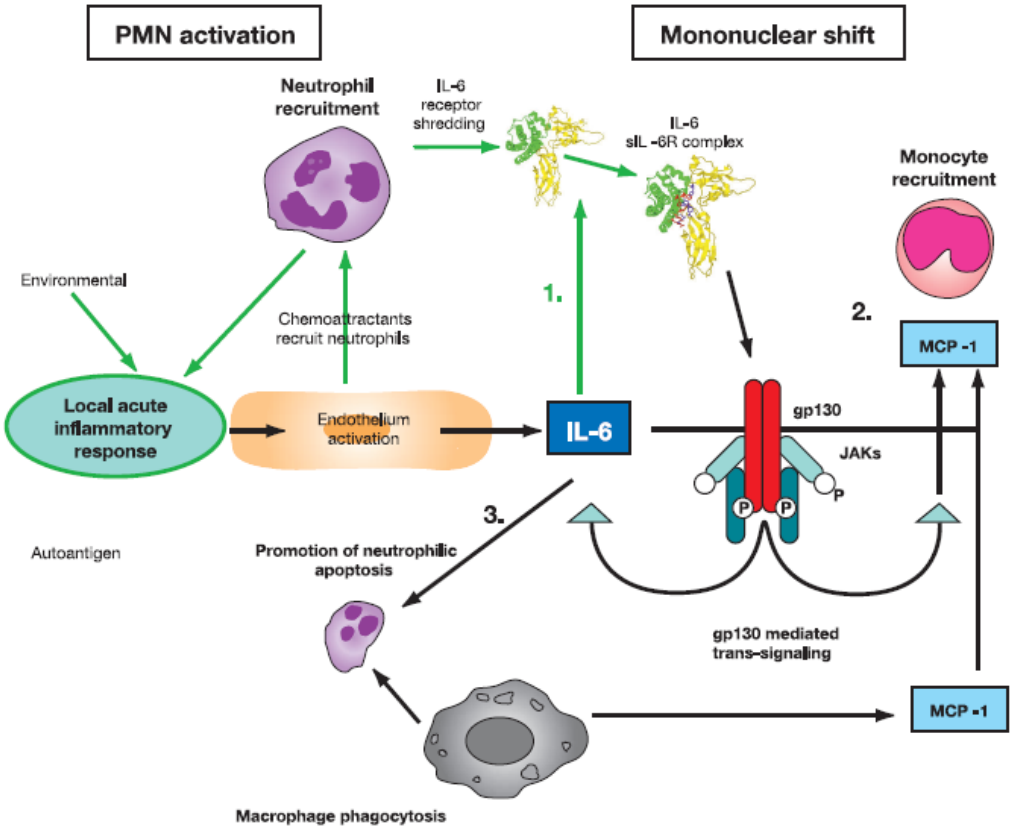

Figure 1.4 The role of interleukin-6 in the development of chronic inflammation (73).

TNF-α is a pro-inflammatory mediator and function in a wide range of biological processes including in infection, autoimmune, inflammation, malignant conditions, regulates cell survival, proliferation, and differentiation as well as controlling an apoptotic and necrotic cell death (74, 75). Activated macrophages, lymphocytes and other cell types, such as smooth muscle cells, adipocytes, endothelial cells, and neuronal tissues generate TNF-□ in a response to bacterial, inflammation (75). Stress and inflammation conditions could activate stress-activated protein kinases (SAPKs) after processing by TNF-□-converting enzyme (TACE), the soluble form of TNF-α is cleaved from trans-membrane TNF-α and mediates its biological activities through binding to its receptor as shown in Figure 1.5. TNF-R1 and TNF-R2 are surface TNF-α receptors and expressed on almost all nucleated cells (76). Previous study reported that TNF-R2 is associated with an increased risk of inflammatory diseases (77).

Overexpression of TNF-α is importance for driving chronic inflammatory and autoimmune disorders such as arthritis, cancer and diabetes mellitus. Nowadays, blocking of TNF-α signaling is wildly uses for the treatment (78) some diseases such as rheumatoid arthritis (RA) (79-81), hearth disease (81, 82), bowel disease (83) and psoriasis (84).

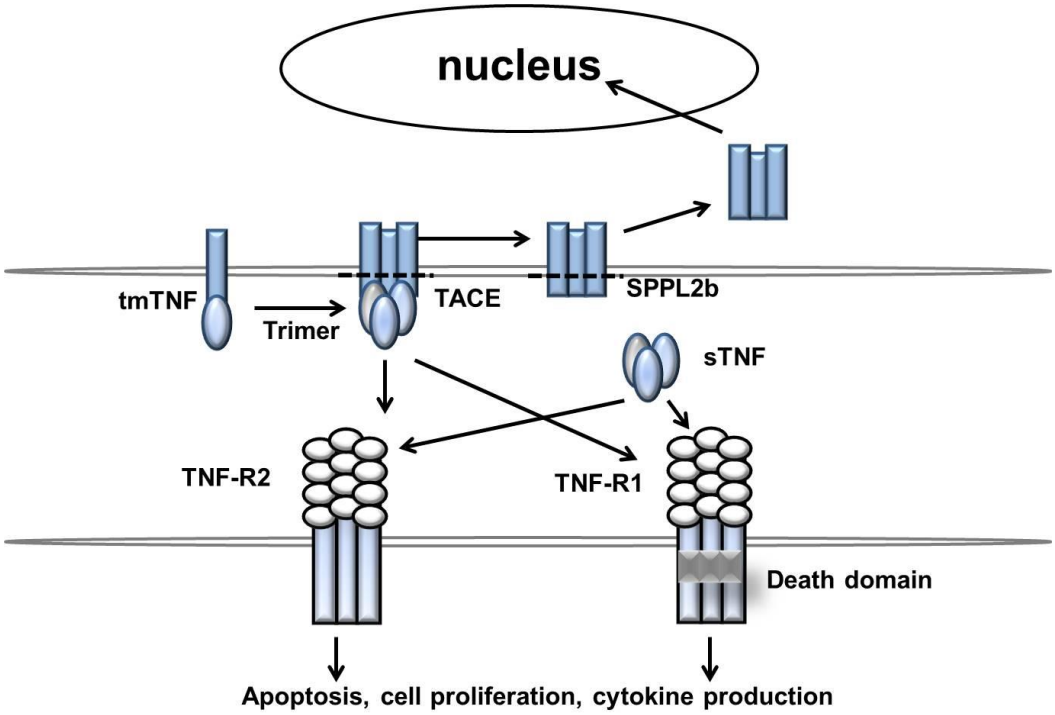

Figure 1.5 Biology of trans-membrane TNF-α and soluble TNF-α (76).

In addition, TNF-α is an important contributor in the development of atherosclerotic lesions by promoting the expression of adhesion molecules on endothelial cells then recruit and activate inflammatory cells (85, 86). Moreover, it can initiate inflammatory cascade inside the arterial wall by interfering metabolism of triglycerides (TG) and glucose during acute inflammatory conditions (87, 88). There is evidence that strongly shows TNF-α increase free fatty acid (FFA) production from adipocyte and liver cells and stimulates lipolysis via TNF-R1 (89, 90). Moreover, TNF-α can suppress the expression of adipocyte triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) then TNF-α might inhibit insulin receptor signaling leading to insulin resistance (91). Furthermore, TNF-α can induce these changes either directly or indirectly, by increasing the level of other

pro-inflammatory cytokines, such as IL-1β and IL-6 that link to chronic inflammation (92, 93).

1.2.3.2.2 Chemokines

Chemokines are a family of small cytokines that can induce chemotaxis in nearby cells (94). Chemotaxis is the directly movement of cells into their surrounding environment by the chemicals stimuli (95). Chemokines are considered as pro-inlammatory molecules, which activate recruitment of inflammatory cells into an infection or injury site during an immune response (95, 96).

Chemokines could stimulate phospholipase A2 enzyme, lead to releasing of arachidonic acid (AA) from breakdown of membrane glycerophospholipids. Oxidation of AA by COX2 can generate pro-inflammatory molecules, leukotrienes, thromboxanes, and prostaglandins (20, 36, 46).

1.2.3.3 Nitric oxide

Nitric oxide (NO) production is associated with elevated of inflammation-associated diseases and increased indices of NO-dependent oxidative stress (39). Low concentration of NO can interact with biological targets commonly in heme-containing protein for the majority of the physiological effects (29). NO usually has a shot half-life in aqueous environment phase as a few seconds. While in a lower oxygen concentration in an environment, NO is stable, its half-life is more than 15 seconds, and thus NO is rapidly diffuse into the cells or neighboring cells through the cytoplasm and plasma membranes. In addition, NO can binds with the heme group of soluble guanylyl cyclase to generate cGMP from GTP. Then, activated cGMP binds on target proteins such as protein kinases, transcription factors and phosphodiesterases and effects to downstream signalings.

Furthermore, NO can react with reactive nitrogen oxide species (RNOS), which is derived from the coupling between NO and either O_2 or O_2^-. Reactive oxygen species (ROS) including superoxide ($O_2^{\bullet -}$), hydrogen peroxide, and peroxynitrite ($ONOO^-$) are also involved in mediating cellular functions and play specific roles in pathogenesis (40, 97, 98). Inflammatory cells produce both the

superoxide anion and nitric oxide during the oxidative stress that leads to inflammation (99-101). Especially, peroxynitrite which generated by the combination of NO and superoxide is a potent oxidizing agent that can cause DNA damage leading to mutation (38, 40, 102-104).

NO is produce by an enzyme called nitric oxide synthase (NOS) from L-arginine. NOS is divided into tree isoform, which consist by an oxygenase and a reductase domain. Neuronal or brain NOS (n/bNOS) and endothelial NOS (eNOS) are constitutively expressed in cells (105, 106). They synthesize NO in response to an increase in calcium and shear stress. During expose to stimuli, such as the inflammatory cytokines and LPS, inducible NOS (iNOS) are up-regulated for a longer period (107). Besides, NO is a highly reactive molecule and rapidly reacts with oxygen to form nitrite and nitrate (Figure 1.6).

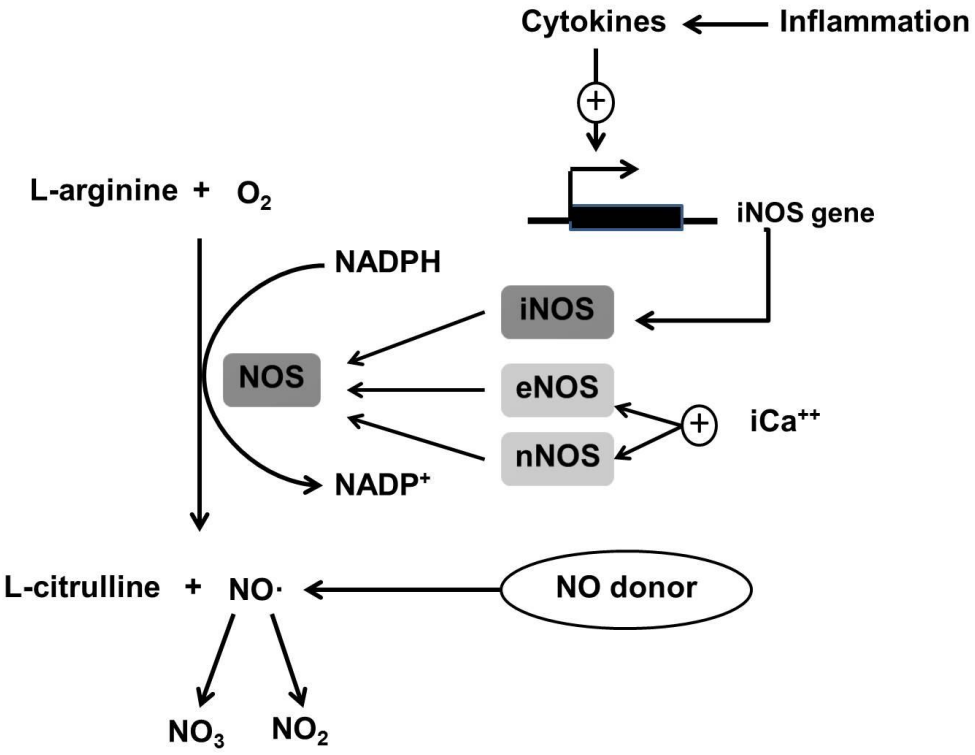

Figure 1.6 The L-arginine-nitric oxide pathway (108).

1.2.3.4 Free radicals and inflammation

Free radicals, molecules with an unpaired electron, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in the development of tissue damage and pathological events (109, 110). The unpaired electron is highly reactive to pair with another free electron (41). ROS can be act as secondary messengers in intracellular signaling cascades, which induce and maintain the inflammation (111). ROS at low concentration shows effect on induce mitogenic response whereas high concentration of ROS could induce cells damage via the oxidation of lipids, proteins and nucleic acids (112). In host defense mechanism, there is a balancing of ROS/RNS by the anti-oxidant of enzymatic and non-enzymatic system.

ROS could activate MAPK signaling via transduction of cytokine/growth factor receptor, non-receptor tyrosine kinases (Src kinase), Ras, PKC and NADPH oxidase leading to up-regulation of several redox-regulated transcription factors

(AP-1, NF-κB, p53, HIF-1, NFAT) signal transduction pathways, which in turn lead to the transcription of genes involved in abnormal in cell growth regulatory pathways.

Oxidative stress has been reported to associate with chronic inflammation and severe metabolic dysfunctions, which ultimately lead to pathogenesis of many human diseases. For example, the relationship between chronic inflammation and rheumatoid arthritis (RA) has already been recognized. Chronic inflammation is a prolonged pathological condition characterized by mononuclear immune cell infiltration, tissue destruction and fibrosis due to excess production of reactive oxygen and nitrogen species (ROS and RNS) as well as toxic free radicals and related with many diseases (Figure 1.7). In the review of A Bala and PK Haldar (2013) have been demonstrated that oxidative stress is the ultimate potential biomarker for determining disease activity in patients with RA (113). Higher amounts of ROS and RNS have been reported in the synovial joints as well as peripheral blood inflammatory cells of the RA patients. The disease is consistently associated with an increase in various pro-inflammatory factors that include cytokines (IL-1β, IL-6 and TNF- α), prostaglandins, reactive oxygen species (ROS) and nitric oxide (NO) at sites of inflammation, coupled with very low concentrations of superoxide dismutase (SOD) in the synovial fluid. In addition, in the report of Taibur Rahman et al. (2012) (114) have been reported that oxidative stress involved in ROS and RNS such as hydrogen peroxide, organic hydro peroxides, nitric oxide, superoxide and hydroxyl radicals are lead to several pathogenesis such as alzheimer's disease (AD) (115, 116), parkinson's disease (PD) (117), coronary heart disease (CHD) (118) and atherosclerosis (119) (120)(Figure 1.8).

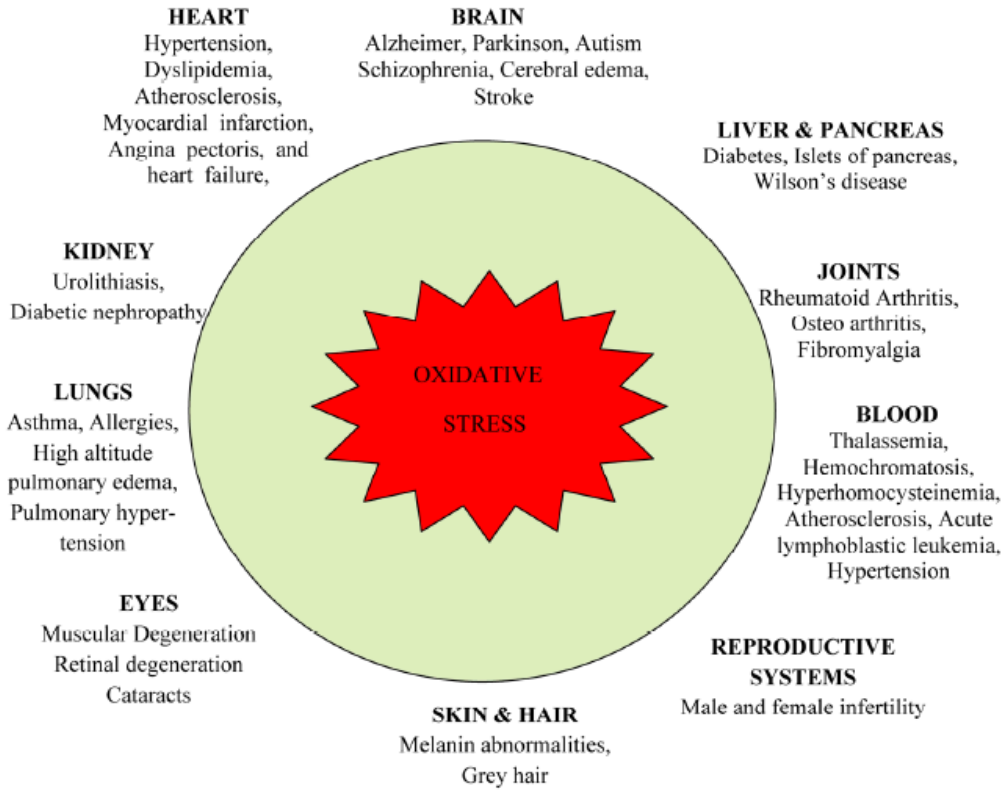

Figure 1.7 Deleterious effects of oxidative stress on human health (109).

Antioxidant enzymes are considered as the first line of cellular defence against oxidative damage. Among the antioxidant enzymes, catalase (CAT), GSH peroxidases (GP), superoxide dismutase (SOD) and antioxidant nutrients are the most important antioxidant defence components in cells exposed to oxygen, whereas reduced glutathione (GSH) is the most important non enzymatic antioxidant defence ROS (121, 122). In addition, non-enzymatic antioxidants are found in food (123) such as ascorbic acid (Vitamin C) (124-126), gamma-tocopherol (Vitamin E) (125-127), glutathione (GSH) (128), carotenoids (129, 130), flavonoids (131, 132), and other (126, 133). These compounds are a well-known as antioxidant or free radical scavenger. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health. However, over production of oxidants is leading to several pathogenesis.

1.2.4 Lipopolysaccharide

Lipopolysaccharide (LPS) is a component on outside membrane of gram negative bacteria such as Escherichia coli, Salmonella enterica, Neisseria meningitidis, *Haemophilus influenzae*, *Bordetella pertussis*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Legionella pneumophila* and *Chlamydia trachomatis* (134, 135). Structure of LPS is shown in figure 1.8.

LPS act as an endotoxin, which containslipid and carbohydrate, and hence the term 'lipopolysaccharide'. It is extremely heat-stable amphiphilic molecule and is a compose of a predominantly lipophilic region, lipid A and a covalently linked hydrophilic poly- or oligosaccharide portion (134). The structure of LPS is containing the membrane-anchoring lipid A domain and a covalently linked polysaccharide or oligosaccharide portion. Lipid A is the highly hydrophobic and endotoxically active part of the molecule. Structurally, lipid A is typically composed of a -D-GlcN-(1-6)-α-D-GlcN disaccharide that carry two phosphoryl groups (at positions 1 and 4'). The structure of lipid A contains four acyl chains by ester or amide linkage. These chains can constitute by fatty acids up to seven acyl substituents. The number and lengths of acyl chains and the phosphorylation state of the disaccharide backbone of LPS is a major contributing factor to endotoxicity. The O-polysaccharide region of bacterial endotoxins is extremely variable whereas some regions are highly conserved between different strains and species. The inner core is characterized by more unusual sugars, particularly Kdo and heptose and is less variable like in the outer core. The outer core typically consists of common hexose sugars. O-Specific chains are present only in smooth-type Gram-negative bacteria. They consist of repetitive subunits which make polysaccharides extending out from the bacteria (136-140). It can bind with host during infection. These chains can help the bacteria to escape the lytic action of the complement complex and also protect the bacteria from the effect of numerous antibiotics.

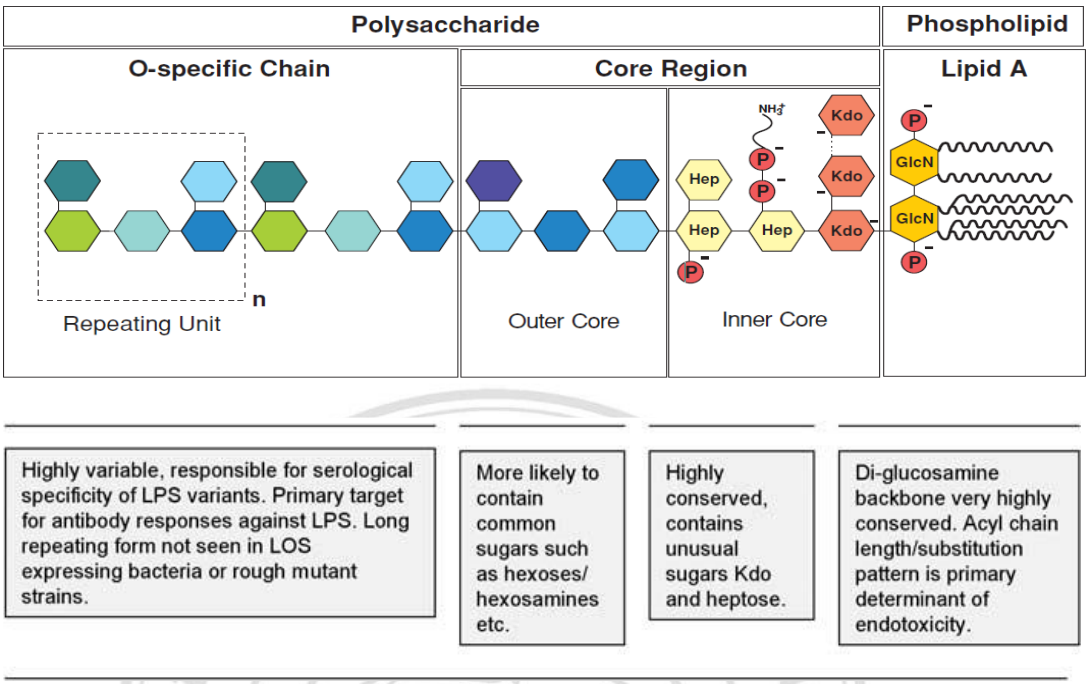

Figure 1.8 Structure of LPS from Gram-negative bacteria (136, 141).

1.2.4.1 The mechanism of lipopolysaccharide (LPS) induced inflammation

LPS is commonly inducing several biological effects in various eukaryotic organisms. The predominant target of LPS in mammalian species is stimulating innate immunity, peripheral monocytes, tissue macrophages and neutrophils, which constitutively express the membrane bound form of the CD14 antigen (mCD14) as well as Toll like receptor-4 (TLR4). Figure 1.9 shows LPS as an "endotoxin" can activates peripheral monocytes or tissue macrophages (Mo/Mφ). LPS binding protein (LBP) catalyzes a transfer of monomerized LPS from aggregate structures to bind with membrane-bound CD14 (mCD14) on the phagocyte surface that induce release of a variety of endogenous mediators via TLR4*MD-2 including lipid mediators such as platelet-activating factor (PAF), thromboxane A2, leukotriene C4 and PGE2, and ROS such as superoxide anion (O_2^-), hydroxyl radicals (•OH) or nitric oxide (NO). In addition, it can activate macrophage to releases pro-inflammatory cytokines such as TNF-α, macrophage migration inhibitory factor (MIF), interleukins IL-1β, IL-6, IL-8, IL-12, IL-15, and IL-18, the colony-stimulating factors M-CSF, GCSF, and GM-CSF (141).

In normal situation, host defense mechanism system can balanced the concentrations of these endogenous mediators by induce fever to recruit macrophage cells to remove dangerous stimuli and reduced oxygen species lead to activation of general anti-microbial, anti-viral and anti-tumoral defense mechanisms In contrast, in pathological situations found high levels of endogenous mediators.

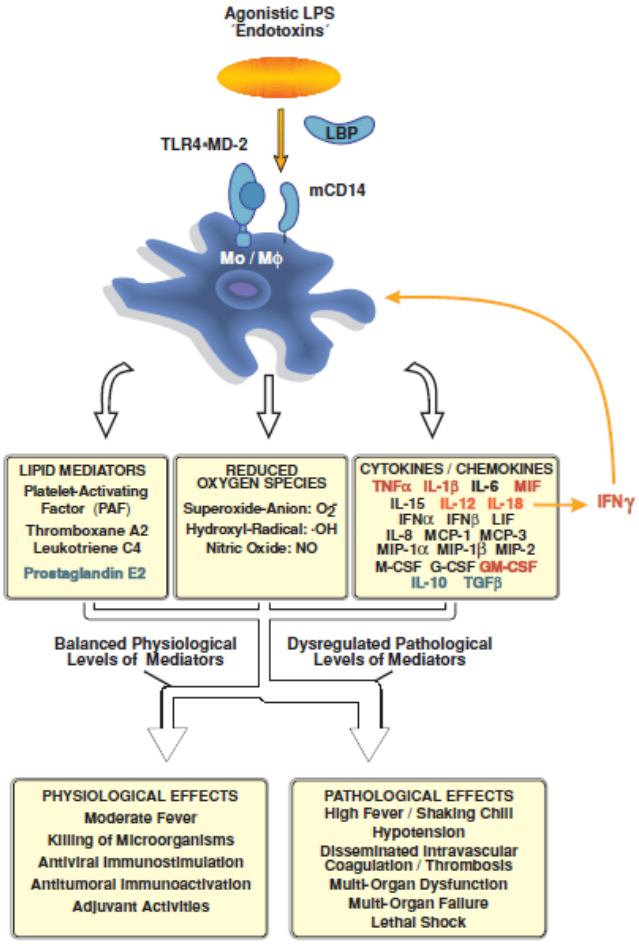

Figure 1.9 LPS-induce innate immune in macrophage cells (141).

Several studies also used LPS to induce inflammation (141). In addition, LPS-stimulated macrophages mediate the inflammatory response by releasing NO, pro-inflammatory cytokines i.e. TNF-α), IL-6 and IL-1β, which are related to pathogenesis diseases. Recently, strongly evidence have been shown that lipid A on LPS could induce the mammalian complement system and could bind with toll-like receptors and then send signals into the cell to stimulate the inflammatory response provoked (142). LPS can stimulate inflammation process by induce production of many proteins including iNOS, COX-2, PGE2, MMP and pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 (143). Several studies found LPS can promote gene expression via MAPKs signaling pathway, ROS signaling pathway, activation of NF-κB, interferon regulatory factors-3 (IRF3) and activating protein-1 (AP-1) transcription factors and increase synthesis of inflammatory mediators (144, 145).

1.2.5 Inflammation and diseases

Inflammation is associated with many diseases including, Alzheimer's disease, Parkinson's disease, cancer, multiple sclerosis, rheumatoid arthritis, and metabolic syndrome especially diabetes mellitus. Activation of pro-inflammatory cytokine signaling pathway occurs in several pathogenesis.

1.2.5.1 Inflammation and cancer

It has been found that inflammation is associated with the development of cancers. Chronic inflammation which is characterized by sustained tissue damage, damage-induced cellular proliferation, and tissue repair is leading to cellular dysfuction that promotes cancers development during initiation, promotion and progression steps. In the initiation step, inflammation induced mutagenic by alteration of various inflammatory related genes such as free radical and nitric oxide (NO) that produced peroxynitrite, which react with DNA that cause accumulation of oncogenic mutations (98, 146). Tumor promotion and progression steps have been reported to be related with inflammatory environment (147, 148). The recruitment of immune cells to the site of inflammation and releasing of pro-inflammatory mediators including, COX-2, iNOS, MMP-2, MMP-9, NO, ROS, IL-1β, IL-6, TNF-α and chemokines is linked inflammation to cancers (10, 12). The tumor inflammatory microenvironment could destroy

basement membrane, which is a process required for invade and migrate of tumor cells. Moreover, pro-inflammatory mediators could enhance cancer cell growth, proliferation, angiogenesis and invasion (11). Therefore, the prevention and inhibition of the chronic inflammation may reduce risk factors of carcinogenesis.

1.2.5.2 Inflammation and insulin resistance

Obesity is a common metabolic disorder worldwide. Excess fat in adipose tissue is accumulated that result in a risk of metabolic syndromes, including insulin resistance. It is a stage of low grade chronic inflammation leading to the increasing of C-reactive protein (CRB), pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), intracellular stress and activation of IKKβ/NF-κB and JNK signaling pathways (56, 149). In addition, pro-inflammatory mediators releases from adipocyte and macrophage could be found in the plasma/serum of obese individual and then raised up in the type 2 DM that associated with insulin-resistance (150, 151). Among inflammatory markers, several studies are reported that TNF-α is involved in the pathogenesis of insulin resistance. As shown in figure 1.10, binding of TNF-α to the TNF-α-receptor, it can inhibit the expression and translocation of glucose transporter 4 (GLUT4) leading to an impairment of the glucose uptake into the cells. Besides, TNF-α could inhibit the insulin-stimulated tyrosine kinase activity of the insulin receptor and the insulin receptor substrate-1 (IRS-1) that involved in insulin signaling pathway. Moreover, TNF-α could stimulate lipolysis in the adipose tissue and then FFA would be increased in the serum. TNF-α also up-regulates monocyte chemotactic protein-1 (MCP-1) expression leading to an accumulation of macrophages in the adipose tissue, that contributes to inflammation-induce trigger insulin resistance (150).

Pro-inflammatory cytokines are overexpressed in obesity, leading to the hypothesis that obesity and inflammation are linked. The impairment of the adipose tissue leads to the release of pro-inflammatory cytokines which induces the inflammatory processes via the auto/ paracrine and endocrine systems. This occurs particularly in intra-abdominal fat and develops into adipose tissue (AT) dysfunction. Interaction of genetic, behavioral and environmental factors are causes of impairment of AT function, which lead to adipocyte hypertrophy,

ectopic fat accumulation, hypoxia, AT stresses, impaired AT mitochondrial function and cause inflammatory processes within adipose tissue (152). During obesity, adipocyte would be enlargement that cause reduced the blood supply to adipocytes resulting in hypoxia (153). Adipose tissue hypoxia may result in disturbances in adipokine secretion and increased macrophage infiltration in adipose tissue. Moreover, during hypoxia, adipocytes would be necrosis leading to macrophage infiltration into adipose tissue. These situation leads to an excess of ROS and pro-inflammatory cytokines that can induce chronic inflammation leading to insulin resistance in adipocytes (153). TNFα, IL-6, IL-1β, and possibly other cytokines and macrophage-secreted factors exert paracrine effects to activate inflammatory pathways within insulin target cells (154). This leads to activation of Jun N-terminal kinase (JNK), inhibitor of κB kinase (IKK)β, and other serine kinases (155). In an insulin-resistant state, JNK1 and IKK signaling is upregulated in insulin-resistant skeletal muscle, fat, and other tissues in humans and rodents (156). This is important, as these serine kinases activate transcription factor targets, including activator protein1(AP) (c-Jun/Fos) and nuclear factor-κB (NF-κB), which then stimulate transcription of an overlapping set of inflammatory pathway genes. These serine kinases can also phosphorylate insulin receptor substrate (IRS) proteins, insulin receptors, and possibly other insulin signaling molecules (156). These serine phosphorylation events interfere with normal insulin action, creating a state of cellular insulin resistance.

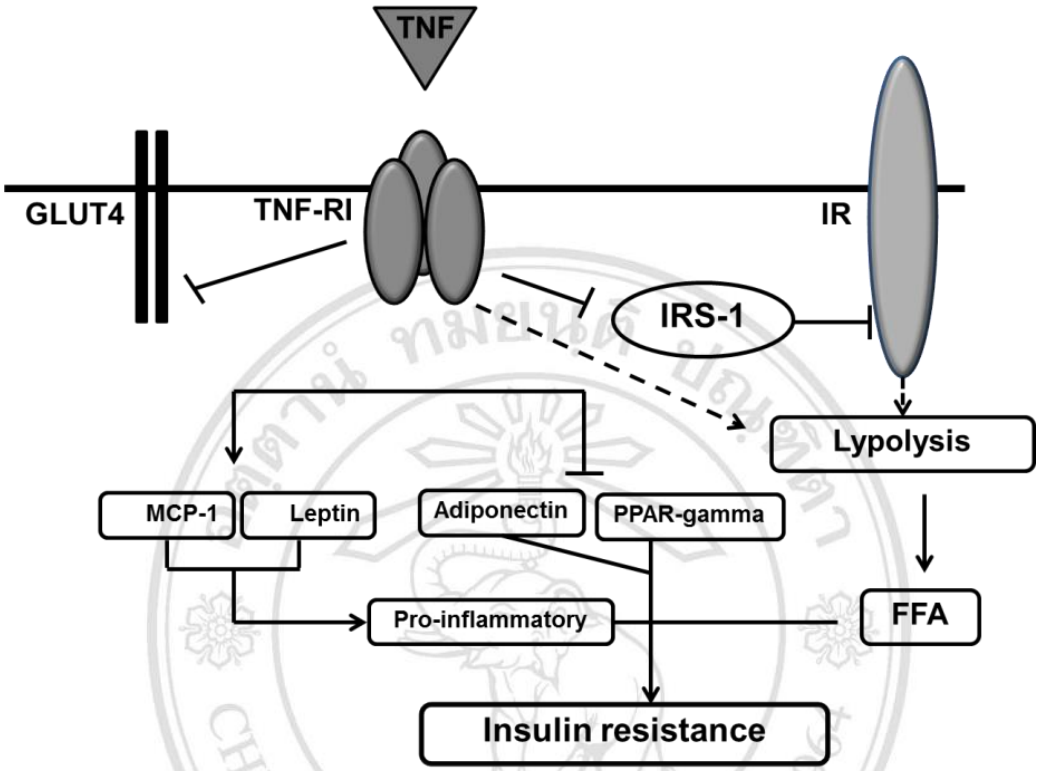

Figure 1.10 Mechanisms of TNF-□ on glucose metabolic pathways (157).

1.2.6 Protection of insulin resistance by anti-inflammation

Recently, cellular mechanism linkage between inflammation and insulin resistance has been established. Prevention and reduction of inflammation are a promising strategy in several chronic diseases. Using of anti-TNF-α blocker has been found to improve insulin resistance in patients with rheumatoid arthritis (158). Moreover, other report is reported that TNF-α antagonist could improve insulin sensitivity by reduction of plasma glucose and increasing adiponectin in obese patients (159). Additional, inhibition of IL-6 signaling pathway could recover insulin sensitivity in humans with immunological disease (160). Blocking of IL-1 signaling pathway can reduce level of systemic inflammation markers and improve glycaemia and β-cell secretory function in type 2 diabetes mellitus (161-163). Consequently, anti-inflammation agents become a promising therapy for treatment of obesity-related insulin resistance.

1.2.7 Thai medicinal plant with inhibitory effect of inflammation

Recently, 80% of people worldwide were used some part of herbal medicines such as ginger (164), galangal (165), lemon leaf (166), basil (167), lemon grass (168), turmeric (169) and cinnamon (170) for health care, which reported by World Health Organization (WHO). The used of herbal supplements has been dramatically increased over the past 30 years. As dietary supplement, Thai people have used herbal medicines to maintain or improve their health. Thai medicinal plants have been reported to contain several biological effects of their phytochemicals that have anti-mutagenic, anti-oxidative, anti-carcinogenic, anti-hyperglycemic and anti-inflammation, making the plants of potential value in treating various diseases including diseases involve in inflammation. In addition, several natural products such as have been used as folk medicine to prevent or relieve inflammation. Several studies are reported that Thai herb also have an anti-inflammatory activity such as in the study of Tuntipopipat et, al. (2009) found *Limnophila aromatica* (Kyeng), dill, kaffer lime, chili, teaw, mint, sweet basil, and pea eggplant could be suppressed NO and TNF-α production in LPS-activated RAW 264.7 cells (171). In addition, in the study of Kittisrisopit et al. (2010) reported that the ethanolic extracts *Artemisia vulgaris* was ingredients for anti-inflammation of hemorrhoid preparation (172). In the study of Siriwatanametanon et al. (2010) demonstrated that *Gynura pseudochina var. hispida*, *Oroxylum indicum* and *Muehlenbeckia platyclada* as Thai anti-inflammatory remedies (173).

1.2.8 *Anoectochilus* species

Anoectochilus, a member of the Orchidaceae, is a valued plant species in several Asian countries more than 50 species, where it is used for ornamental, culinary, and medicinal purposes. Many species of Anoectochilus such as *Anoectochilus roxburghii*, *Anoectochilus formosanus*, *Anoectochilus elwesii* and *Anoectochilus setaceus* are popular among in china, Vietnam, Sri Lanka and Taiwan because of their unique medicinal properties, such as it effects on clearing heat and cooling blood (174), eliminating dampness, and detoxification (174). For example, the extract of *A. formosanus* showed significant activity in decreasing the levels of the cytosolic enzymes such as LDH, GOT, and GPT (175), and the result demonstrated that *A. formosanus* possessed prominent hepatoprotective activity against CCl4-induced

hepatotoxicity (175). Kinsenoside, an active compound isolated from *A. roxburghii* could reduce blood glucose and cholesterol levels and enhanced the oxidation resistance of diabetic mice induced by streptozotocin (176). In addition, kinsenoside extracted from *A. roxburghii* and *A. formosanus* also reduced the production of nitric oxide and inhibited LPS-induced NF-κB activity by decreased the level of p65 and p50 protein expressions (177). Recently, it has been reported that kinsenoside exhibits significant anti-hyperglycemic effects, reduced chloresterol levels and possesses antioxidant, anti-inflammation in diabetes rat induced by streptozotocin *in vivo* (176, 178). In study of Cai J et al. (2014) found a new triterpene, 3-β -O-olean-11,13 (18)-diene-23,28-dioic acid, derived from A. elwesii which could enhance glucose uptake in insulin-resistant human HepG2 cells (179).

1.2.8.1 *Anoectochilus burmannicus*

A. burmannicus Rolfe (Orchidaceae) (Figure 1.11) is also known in Thailand as nok-kum-fai. *A. burmannicus* is a terrestrial and rather small orchid whose oval flowers have clearly visible veins. This orchid species belong to the jewel orchid because of its pretty leaf. The small flowers of the *A. burmannicus* bloom on October to November and can number two to fifteen on a stalk of about 25 cm in length. Originally the plant grows in the Himalaya Mountain and also is found in Thailand, Bhutan, Laos, China, Vietnam and Indonesia at an altitude of 300-1800 meters, mostly along watersides. The study about the biological effects of *A. burmannicus* has not been reported. *A. burmannicus* may exert similar or different biological effects as other *Anoectochilus* species.

Figure 1.11 *Anoectochilus burmannicus*

1.3 Objectives

1. To prepare aqueous and ethanolic extracts from *A. burmannicus* and to determine the phytochemical constituents of these extract.
2. To determine the anti- inflammatory activity of the extracts in lipopolysaccharide-treated RAW 264.7 macrophage.
3. To determine inhibitory effect of the extracts on TNF-α-induced insulin resistance of 3T3-L1 adipocyte.