CHAPTER I

INTRODUCTION

Macrophages are mononuclear phagocytes derived from granulocyte-monocyte progenitors in the bone marrow. They recognize and destroy invading microganisms by phagocytosis and secretion of various mediators. There are two major groups of macrophage secretory mediators, classified by their killing mechanisms. They are oxygen-dependent and oxygen-independent mediators. The former group includes hydrolytic enzymes and cytokines that involve in oxygen-independent killing mechanisms. The latter are reactive oxygen species (ROS) and reactive nitrogen intermediates that kill microorganisms via oxygen-dependent killing mechanisms (Goldsby et al., 2003). Activated macrophages produce superoxide anion (O_2^-) from a metabolic process known as the respiratory burst. The superoxide anion then converts to the other strong oxidizing agents, such as hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2). Furthermore, activated macrophages also produce nitric oxide (NO) and release various inflammatory cytokines, which regulate immune function (Goldsby et al., 2003; Victor, Rocha, and De la Fuente, 2004). ROS and NO are crucial to eliminate pathogens in the host-defense mechanism. However, these radicals can cause oxidative damages of macromolecule including lipids, proteins and DNA if they are in excess (Dedon and Tannenbaum, 2004). The evidence has been demonstrated in various diseases with hyperactivity of macrophages (Cadenas and Cadenas, 2002; Ratnam et al., 2006; Victor et al., 2004).

NO is a soluble gas that plays a critical role in various biological functions, including the vasodilatation of smooth muscle, neurotransmission, nonspecific immune responses against pathogens, and cell death (Garcia and Stein, 2006). It is synthesized from L-arginine and molecular oxygen by nitric oxide synthase (NOS). There are three isoforms whose tissue distribution and regulation largely determine physiological action. These isoforms are endothelial, neuronal and inducible nitric oxide synthases (eNOS, nNOS and iNOS). The constitutive isoforms, nNOS and eNOS, are calcium-dependent enzymes and lead to a rapid, transient, and low-level production of NO. In contrast, iNOS is not expressed in resting cells, but is induced

upon macrophage activation by cytokines, for example interferon-gamma (IFN-γ) or a component of bacteria cell wall such as lipopolysaccharide (LPS). There is a lag time, usually several hours, between cell activation and NO production corresponding to mRNA and protein synthesis. iNOS catalyzes high levels of NO production, which is sustained for hours or longer, depending on the expression of the enzyme in the cells or tissues (Garcia and Stein, 2006; Coleman, 2001).

Besides NO production, macrophages generate various oxidative substances such as hydroxyl radicals (OH$^-$), hydrogen peroxide (H_2O_2) and hyperchlorite (ClO$^-$) for destroying pathogens. These reactive species can react with superoxide anion to generate peroxynitrite (ONOO$^-$). Peroxynitrite is a strong oxidizing and cytotoxic product. Furthermore, NO and these reactive species can activate cellular signal transduction pathways to synthesize inflammatory cytokines such as interleukins (IL-1, IL-4, IL-6, IL-8) and tumor necrosis factor-alpha (TNF-α). These cytokines can stimulate specific immune components, including T-cells and B-cells (Coleman, 2001; Garcia and Stein, 2006).

Under normal conditions, macrophages can produce various enzymatic antioxidants, including superoxide dismutase (SOD) and catalase, and endogenous antioxidant scavenging compounds, such as reduced glutathione (GSH) and vitamins C and E, to detoxify reactive oxygen species. The excessive production of reactive species cause oxidative stress and can damage macrophages themselves as well as the surrounding host tissues, leading to oxidation, nitration, halogenation, and deamination of biomolecules including lipids, proteins, carbohydrates, and nucleic acid. This results in the formation of toxic and mutagenic products (Dedon and Tannenbaum, 2004). The oxidative stress can induce various diseases such as autoimmune neuropathy (Kiefer et al., 2001), atherosclerosis (Jara et al., 2006), rheumatoid arthritis (Paulos et al., 2004) and sepsis (Victor et al., 2004. In these disease conditions, total antioxidant capacity is decreased and the oxidant load is increased. Furthermore, this redox imbalance can also activate inflammatory cytokines that lead to excessive stimulation of macrophages (Victor et al., 2004).

The exogenous antioxidants have been used to decrease the harmful effect of the oxidative stress (Cadenas and Cadenas, 2002; Ratnam et al., 2006). The in vitro

modulating effects of two commonly used antioxidants, N-acetylcysteine (NAC), and α-tocopherol (TOC) on macrophage functions have been reported. NAC is currently used as a mucolytic agent and in the treatment of acetaminophen-induced hepatotoxicity. It directly reacts with free radical and replenishes intracellular GSH. An adequate production of GSH controls and regulates inflammatory processes by influencing nuclear factor-kappa B (NF-κB) activation. This results in decreased NO production (Pahan et al., 1998) and lower TNF-α release (Victor, Rocha, and De la Fuente, 2003; Mendez, Garcia, and Maier, 1995). It was reported that NAC protected macrophages against triacylglycerol-induced lipotoxic effect (Aronis, Madar, and Tirosh, 2005). TOC, a potent peroxyl radical scavenger, is a chain-breaking antioxidant that prevents membrane peroxidation (Traber and Packer, 1995). TOC has shown several effects on oxidative stresses in the macrophages, including inhibition of NO production in smokeless tobacco-induced macrophages (Hassoun et al., 1995) and inhibition of TNF-α and prostaglandin E_2 production in endotoxin-induced alveolar macrophages (Mendez et al., 1995). Some scientific evidence indicates that use of antioxidant may also work in vivo (Victor et al., 2003). Utilization of antioxidants to modulate macrophage functions, however, requires efficient intracellular delivery of antioxidant molecules.

However, most of the antioxidants have major drawbacks regarding their delivery to target cells due to their unsatisfactory biopharmaceutical properties leading to poor oral bioavailability and difficulty in intracellular delivery. The biopharmaceutical drawbacks of antioxidants include poor solubility, low permeability, instability, and pre-systemic degradation. The low bioavailability, as well as inefficient intracellular delivery, results in needs of either much higher oral doses or alternative routes of administration such as injection for therapeutic purposes. Thus, formulation of these antioxidants for efficient delivery is still a challenge for pharmaceutical scientists (Ratnam et al., 2006). Hydrophilic antioxidants usually have difficulty entering the cell due to their poor permeability through cell membrane. This can lead to either poor oral bioavailability or inefficient intracellular delivery. Lipophilicity, on the other hand, imposes another problem on lipophilic antioxidants. Formulation of these antioxidants into liquid dosage forms usually requires co-solvents or solubilizing agents in amounts that are sometimes not suitable for parenteral use. Thus, a suitable delivery system that can increase

intracellular delivery of hydrophilic antioxidants as well as help to solubilize lipophilic antioxidants is undoubtedly beneficial for these compounds.

Liposomes are known to increase intracellular delivery of many hydrophilic compounds (Alipour et al., 2007; Stone and Smith, 2004; Stevenson, Baillie and Richards, 1984). Due to their bilayer structure, liposomes can also accommodate lipophilic molecules in their structure, resulting in increased solubility of those molecules (Gulati et al., 1998). They are biocompatibility, biodegradable, and have high potential as drug delivery systems from their amphiphilic characteristics (Ratnam et al., 2006). In addition, they are particulate systems. This renders liposomes to be rapidly recognized by the phagocytes in the reticuloendothelial system (RES), in which macrophages are predominant. Accordingly, liposomes should be beneficial for intracellular delivery of antioxidants into macrophages.

Many researchers investigated the effect of liposome component on phagocytosis in macrophages. The rate of phagocytosis depends on physicochemical properties of liposome such as size, composition, and surface charge (Oussoren and Storm, 2001). Negatively charged liposomes consisting of phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidic acid (PA) are phagocytosed faster and in greater extent than neutral liposomes by phagocytes (Makino et al., 2003 and Lee, Hong, and Papahadjopoulos, 1992). Moreover, negatively charged liposomes, especially those containing PS, can inhibit NO production via the inhibition of iNOS (Aramaki et al., 1996) and induction of TGF-β, an anti-inflammatory cytokine induction (Matsuno, Aramaki, and Tsuchiya, 2001). Thus, the effect of antioxidant-containing liposomes on NO production might also be modified by the negative charge on the surface of liposomes.

This study was designed to investigate whether incorporation of antioxidants into liposome structure would be more beneficial than free antioxidants in reducing LPS-activated macrophage function. NAC and TOC were used as models for hydrophilic and lipophilic antioxidants, respectively. These antioxidants are known to inhibit NO production in LPS-stimulated macrophages (Pahan et al., 1998; Mendez et al., 1995; Vandana et al., 2006). Effects of negative charges on liposome membrane were also examined with dicetylphosphate and phosphatidylglycerol since these two

negative lipids are commonly used in liposome preparations to prevent aggregation. The results would indicate the feasibility in formulation of antioxidant-containing liposomes as well as the possibility of utilizing such liposomes to modulate macrophage functions.

The specific objectives of this study were to determine:

1. The effects of incorporation of lipophilic and hydrophilic antioxidants into liposomes on modulation of LPS-stimulated macrophage activity

2. The effects of liposome composition and negative surface charge on LPS-stimulated macrophage activity