CHAPTER V

DISCUSSION

Many studies indicate that overproduction of cytokines and nitric oxide (NO) leads to the development of oxidative stress (Cadenas and Cadenas, 2002; Victor et al., 2004). Macrophages, which produce proinflammatory cytokines and NO, are involved in the cellular functions of the body that play an important role in the inflammatory process. Over-activity of macrophages can cause oxidative stress that is harmful to surrounding tissues, leading to inflammatory diseases such as rheumatoid arthritis and atherosclerosis, cancerous diseases and premature aging (Chellat et al., 2005; Ratnam et al., 2006). Moderation of macrophage activity in these diseases is well accepted to have significant therapeutic value (Chellat et al., 2005). Macrophages can be activated by various cytokines and components of bacteria cell wall such as lipopolysaccharide (LPS) to produce NO (Goldsby et al., 2003). In order to evaluate whether liposomes would be of value in delivery of antioxidants directly to macrophage cells to modulate their activity, inhibition of NO production in LPS-stimulated macrophages was used as a feasible indicator in the present study. The results of this present study demonstrated that LPS in moderate concentrations (0.125-2 μg/ml) could activate J774A.1 murine macrophage cells to increase their NO production significantly. The extent of activation varied with seeding density. Intracellular pathways for LPS-stimulated macrophages that lead to NO production are believed to involve tyrosine kinase activation, phosphorylation and translocation of the transcription factor NF-κB (Victor et al., 2003). Various LPS concentrations (10 ng/ml to 10 μg/ml) have been used for stimulating macrophage to induce NO production in the literature (Akifumi et al., 1998; Mendez et al., 1995; Aramaki et al., 1996). The effective concentration of LPS depends partly on experimental conditions including seeding density as well as cell types (Pahan et al., 1998).

α-Tocopherol (TOC) is a lipid-soluble antioxidant that is important in the protection of cells undergoing lipid peroxidation from oxidative stress. TOC functions as a direct scavenger of reactive oxygen intermediates (Mendez et al., 1995). TOC has been study as an antioxidant that can inhibit inflammatory cytokines and NO

production (Mendez et al., 1995; Vandana et al., 2006). Mendez et al. (1995) reported that exposure of TOC (50-1000 μg/ml, equivalent to 0.116-2.32 mM) for 4 hours inhibited TNF production, accumulation of TNF messenger RNA, procoagulant expression and prostaglandin E_2 production in LPS-stimulated alveolar macrophages. Reduction of TNF can lead to reduction in NO production (Victor et al., 2004). Though there is no direct report on the effect of TOC in NO inhibition in LPS-stimulated macrophages, NO production is inhibited by TOC in chromium induced oxidative stress in murine macrophage cells (Vandana et al., 2006). In this present study, TOC could inhibit NO production by LPS-stimulated J774A.1 cells in a concentration dependent manner. However, cell viability was compromised at the concentration of 1 mM. The cytotoxicity seen at comparatively lower concentration than those used in the other study might be the result of longer contact time used in this present study (24 hours versus 4 hours).

The antioxidant activity of *N*-acetylcysteine (NAC) is in part due to its ability to donate cysteine groups (Victor et al., 2004). Consequently, NAC acts as a substrate to increase the endogenous intracellular antioxidant glutathione. NAC itself can also function as a direct reactive oxygen intermediate scavenger (Ruben, Katelijne and Arnold, 1997). The results of this present study clearly showed that NAC at 10-20 mM could inhibit LPS-stimulated NO production in J774A.1 cells, which was in good agreement with earlier studies in macrophage cells. Pahan et al. (1998) reported that 20 mM of NAC significantly decreased NO production and induction of iNOS and inhibited activation of NF-κB in LPS-stimulated peritoneal macrophages. Mendez et al. (1995) reported that exposure of alveolar macrophages to NAC (0.1-10 mM) for 4 hours inhibited TNF-α production. The inhibitory effect of NAC on LPS-stimulated macrophages is due to the decrease in ROS that modulate the intracellular signal pathway for the activation of NF-κB (Pahan et al., 1997; Victor et al., 2004).

Negatively charged liposomes containing phosphatidylserine (PS) or phosphatidic acid (PA) are preferentially taken up by phagocytic cells such as macrophages (Lee et al., 1992; Makino et al., 2003; Allen et al., 1991). Moreover, the negatively charged liposomes composed of PA and PS (PC:CH:PS or PA, 1:1:1 molar ratio) at 500 μg/ml phospholipid concentration were reported to show significant inhibitory effect on NO production, whereas a much smaller effect was seen with

liposomes without negative charges (PC:CH, 2:1 molar ratio) (Aramaki et al.,1996). The mechanism of inhibition was proposed to be due to suppression of iNOS induction. In this present study, neutral (PC:CH, 70:30 molar ratio) and both negatively charged liposomes (PC:CH:DCP or PG, 60:30:10 molar ratio) gave comparable results on NO production. The extent of inhibition in this present study was much lower than the extent of inhibition seen with PS- and PA-containing liposomes in the previous study. The discrepancy seen here could be due to the differences in both the type (PS and PA versus DCP and PG) and the amount (33 versus 10 mol%) of negatively charged lipids used between the two studies. DCP and PG were investigated in this present study since the costs of these two lipids are not as prohibitory as those of PS and PA. Thus, it would be more feasible to formulate liposomes as drug delivery systems using DCP or PG. Much effort has been put toward elucidation of the underlying mechanism of inhibitory effects of liposomes on NO production in LPS-stimulated macrophages (Aramaki, Matsuno and Tsuchiya, 2001). Negatively charged liposomes containing PS can down-regulate NO production in LPS-stimulated macrophages through the induction of the transcription factor TGF-β (Matusno et al., 2001). NO production, iNOS mRNA expression and iNOS production are all inhibited by TGF-β. However, it is still unclear how the whole cascade operates.

From the scientific evidence that both antioxidants and blank liposomes can inhibit NO production, a synergistic or at least an additive effect of antioxidants and liposomes on NO production in LPS-stimulated macrophages was expected. Surprisingly, antioxidants in the presence of liposomes, either being incorporated in the liposome structure or co-existing with blank liposomes, decreased the intrinsic inhibitory effect on NO production of blank liposomes. One possible explanation could be that the antioxidant might interfere with the inhibitory process of liposomes on NO production. Upon phagocytosis of liposomes, macrophages become activated (Goldsby et al., 2003). Activated macrophages can produce NO, reactive oxygen species and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) (Alberts et al., 2002). These mediators, especially NO, can control the process of immune response by a negative auto-regulatory feedback including inhibition of TNF-α synthesis (Iuvone et al., 1996), inhibition of the inducible nitric oxide synthase

(iNOS) (Hinz, Brune and Pahl, 2000) and induction of the expression of anti-inflammatory cytokine transforming growth factor-beta (TGF-β) (Ayache et al., 2002). This negative auto-regulation help to protect macrophage cells from auto-destruction. Thus, although NO production was also activated in J774A.1 cells from phagocytosis of liposomes, it was possible that the decrease in overall NO production when the macrophage cells were further stimulated by LPS was seen in this study as a result of negative auto-regulatory feedback. On the other hand, the presence of an antioxidant with liposomes could decrease the overall activation of macrophage function that was the result of phagocytic process. Thus, the auto-regulatory feedback mechanism was less prominent, making the effect of LPS stimulation more clearly seen. To prove that the auto-regulatory feedback really occurred under these experimental conditions, further studies should be carried out. Possible experiments would be to try to prove that pro-inflammatory mediators were really triggered by phagocytosis of liposomes and that antioxidants could suppress these mediators.

In addition, incorporation of TOC into liposome bilayers resulted in neither synergistic nor additive effect on inhibition of NO production. It was expected that after phagocytosis TOC would be released from liposomes and penetrated through the phagosomal membrane into the cytoplasm. However, the results indicated otherwise. In the case of the water-soluble antioxidant NAC, it was expected that entrapment into liposomes would increase the extent of cellular uptake of the antioxidant since phagocytosis would be triggered by the presence of liposome structure. However, since the effect of blank liposomes on NO production was so potent under the conditions used in this study, it obscured any effect that could have resulted from phagocytosis. In addition, an antagonistic effect between these antioxidants and liposomes could also occur as previously discussed.

Though both antioxidants could inhibit NO production in LPS-stimulated J774A.1 cells, the extents of inhibition seemed to be different between the two antioxidants. The difference in antioxidant concentration used in the study might be partly responsible for the discrepancy. On the other hand, since NO production occurs mainly in the aqueous environment of the cytoplasm, lipid-soluble TOC might play a less pronounced role than NAC did on NO production in the cell.

Another unexpected result seen in this study was that NAC-loaded negatively charged liposomes had severe cytotoxic effect on J774A.1 cells. This cytotoxic effect was not seen with NAC-loaded neutral liposomes. In general, macrophage cells can take up negatively charged liposomes more efficiently than neutral liposomes (Lee et al., 1992; Stevenson et al., 1984; Allen et al., 1991). To prove that J774A.1 cells took up negatively charged liposomes better than neutral liposomes, calcein was entrapped as a marker in these liposomes. However, the result with neutral liposomes and DCP-containing liposomes did not show significant difference in the uptake within the first 4 hours of contact time. The negative result could be due to the low content of the negatively charged lipid in the preparation. Lee et al., (1992) reported that negatively charged lipid content of less than 30 mol% had little effect on the uptake of liposomes by J774 cells. The effect was negligible at 9 mol%, which was comparable to the extent of negatively charged lipids used in this study. When a longer contact time was used, calcein uptake was indeed much higher from neutral liposomes than from negatively charged liposomes. Thus, more uptakes of negatively charged liposomes were not the reason behind the cytotoxicity of NAC-loaded negatively charged liposomes. To further investigate whether NAC-containing liposomes were the species responsible for the cytotoxicity, free NAC was excluded from the preparation. The results, however, indicated that this was not the case. Since co-incubation of NAC and blank negatively charged liposomes did not result in cell death, the cytotoxicity could result from the increase uptake of free NAC into the cells due to phagocytosis triggered by NAC-encapsulated negatively charged liposomes. To prove this hypothesis, blank negatively charged liposomes and NAC-encapsulated negatively charged liposomes were co-incubated with calcein solution and calcein uptake were compared. The results indicated that only NAC-encapsulated negatively charged liposomes, but not blank liposomes, might increase uptake of the water-soluble marker into the cells. Thus, the increase uptake of free NAC induced by NAC-encapsulated negatively charged liposomes could be proposed as the cause of cytotoxicity. To prove this proposed mechanism, however, further study with neutral liposomes as a control and with varying contact times should be designed.