CHAPTER V

DISCUSSION

In this study, the effects of normal HDL and AP-HDL on the growth of gram-negative and gram-positive bacteria *in vitro* and on LPS-induced leukocyte adhesion on endothelial cells *in vivo* were examined. The results showed that both normal HDL (50, 100, 200, 400, 800 and 1,670 μg/ml) and AP-HDL (50, 100, 200, 400, 800 and 1,670 μg/ml) had no effects on the growth of *E. coli*, gram-negative bacteria, and *S. epidermidis*, gram-positive bacteria. However, our study showed that both normal HDL (10 and 20 μg protein of normal HDL/100 g BW) and AP-HDL (5 and 10 μg protein of AP-HDL/100 g BW) were able to inhibit LPS-induced leukocyte adhesion on endothelial cells *in vivo*. Interestingly, AP-HDL was more effective than normal HDL. Since lower concentrations of AP-HDL (5 μg protein of AP-HDL/100 g BW) were required to completely inhibit LPS-induced leukocyte adhesion. This inhibitory effect of HDL was not a direct effect on endothelium but it required interaction between HDL and LPS. In addition, we identified that the protein, not lipid, component of HDL was responsible for this effect. Apo A-I, the major protein of HDL, could, in fact, inhibit LPS-induced leukocyte adhesion on endothelial cells.

Effects of normal HDL and AP-HDL on the growth of gram negative and gram-positive bacteria.

The present study demonstrated that normal HDL and AP-HDL did not affect the growth of gram-negative *E. coli* (Figure 20&21). Previously, an *in vitro* study showed that human HDL could inhibit the growth of gram-positive *S. epidermidis*. It was proposed that lipids or proteins of HDL might insert into the bacterial cell membrane and result to cell lysis (Tada et al.,

1993). Our study, however, could not demonstrate the antibacterial effect of normal HDL or AP-HDL on gram-negative *E. coli*.

In gram-negative bacteria, the majority of the cell wall consists of the outer membrane, whereas only about 10% of the wall is peptidoglycan. The outer membrane is made of lipopolysaccharide. Many differences in cell wall structure between gram-positive and gram-negative bacteria might expain the different effects of HDL.

Firstly, the outer membrane of gram-negative bacteria is relatively permeable to small molecules even though it is basically a lipid bilayer. Porins are proteins present in the outer membrane of gram-negative bacteria and function as channels for the entrance and exit of hydrophilic low-molecular-weight substances. They are transmembrane proteins containing three subunits which associate to form small membrane holes about 1 nm in diameter (Koneman et al.,1997, Madigan, 2000). The diameter of HDL is about 7 – 12 nm (Tuck, 2002), thus, it is too large to pass through porins into the cells. Secondly, periplasm, the region between the outer membrane and cytoplasmic membrane, is present only in gram-negative bacteria (Madigan, 2000). The periplasm of gram-negative bacteria contains several proteins including hydrolytic enzymes and detoxifying enzymes (Beveridge, 1999). Even if HDL and its proteins could pass through the outer membrane, they might be trapped in the periplasm and destroyed by these enzymes. Thirdly, although several studies have found that HDL could bind LPS (Ulevitch et al., 1978, Weinstein et al., 1979, Ulevitch et. al., 1979, Ulevitch et al., 1981), it is possible that HDL might be trapped on the LPS layer and could not pass the LPS layer to affect the growth of bacteria.

In addition, differences in incubation techniques might explain the discrepancy. Because Tada et al. (1993) incubated HDL and bacteria in a phosphate buffered solution devoid of any nutrient, therefore, the growth of bacteria was not supported and could be easily inhibited. We, however,

incubated HDL and bacteria in tryptic soy broth which supports the growth of bacteria and it may be more difficult to show the inhibition.

This study found that normal HDL and AP-HDL could not inhibit the growth of gram-positive *S. epidermidis* (Figure 22&23). However, Tada et al. had shown that normal HDL could inhibit the growth of *S. epidermidis* (Tada et al., 1993) and suggested that proteins or lipids of HDL might insert themselves into the bacterial cell membrane, causing the cell lysis. Similarly, this discrepancy might be caused from the difference of incubation techniques described above.

Effects of normal HDL and AP-HDL on LPS-induced leukocyte adhesion on endothelial cells.

Our study found that normal HDL and AP-HDL could significantly inhibit LPS-induced leukocyte adhesion on endothelial cells in a concentration-dependent manner (Figure 25&26). AP-HDL (5 μg protein of AP-HDL/100 g BW) appeared to be more effective than normal HDL because lower concentrations of AP-HDL were able to completely inhibit LPS-induced leukocyte adhesion on endothelial cells (Figure 27). Besides, HDL alone did not affect the endothelial cells (Figure 28) and the inhibitory effect of HDL required the incubation between HDL and LPS (Figure29).

LPS is a membrane lipid of gram-negative bacteria that acts as a potent inflammatory stimulus in humans and other mammals. *In vitro*, LPS can stimulate adhesion molecule expression of endothelial cells by stimulating cytokine and chemokine release from several cells types, including monocytes, macrophages, and endothelial cells (Mantovani et al., 1989, Pugin et al., 1993, Haziot et al., 1993, Schletter et al., 1995, Jiang et al., 2000, Chow et al., 1999, Pugin et al., 1993, Dentener et al., 1993). *In vivo*, LPS is able to stimulate endothelial adhesion molecule expression (Yipp et al., 2002) and leukocyte adhesion on endothelial cells (Davenpeck et al., 1998, Woodman et al., 2000).

In figure 25&26, normal HDL (10 μg/LPS 0.1 μg/100 g BW) and AP-HDL (5 μg/LPS 0.1 μg/100 g BW) could completely attenuate LPS-induced leukocyte adhesion on endothelial cells. Lower concentration of HDL, however, were not sufficient to inhibit LPS-induced leukocyte adhesion probably because there were some unbound LPS left to induce leukocyte adhesion on endothelial cells. AP-HDL (5 μg protein of AP-HDL/100 g BW) was more effective than normal HDL since lower concentration of AP-HDL was able to inhibit LPS-induced leukocyte adhesion on endothelial cells. It is possible that APR induced the changing of AP-HDL composition, proteins and lipids, (Khovidhunkit et al., 2000) resulting in the inhibitory effect of AP-HDL more effective than normal HDL function. The observed inhibition was due to the interaction between HDL and LPS, rather than the direct effect of HDL on endothelial cells, because it required incubation of LPS with HDL and HDL alone did not affect the endothelial cells. It is possible that HDL binds LPS and causes its conformational change into an inappropriate shape for the LPS receptor on endothelial cells. Previously, it was suggested that HDL diminished the ability of LPS to stimulate macrophage cytokine production because HDL formed complex with LPS resulting in less interaction of the LPS and the receptor on the macrophage surface (Cavaillon et al., 1990). Brandenburg K. et al. studied the interaction of HDL with LPS and lipid A by a variety of physical techniques and biological assays. They found that the functional groups of LPS interacting with HDL were the phosphates and the diglucosamine backbone. In addition, LPS binding to HDL show a change of a unilamellar/inverted cubic into a multilamellar structure of lipid A (Brandenburg et al., 2002).

Since HDL is composed of two major components, proteins and lipids. The observed inhibitory effect of HDL might be due to either component of HDL.

The effects of the molecular components (apoHDL, lipids and apo A-I) of normal HDL and AP-HDL on LPS-induced leukocyte adhesion on endothelium

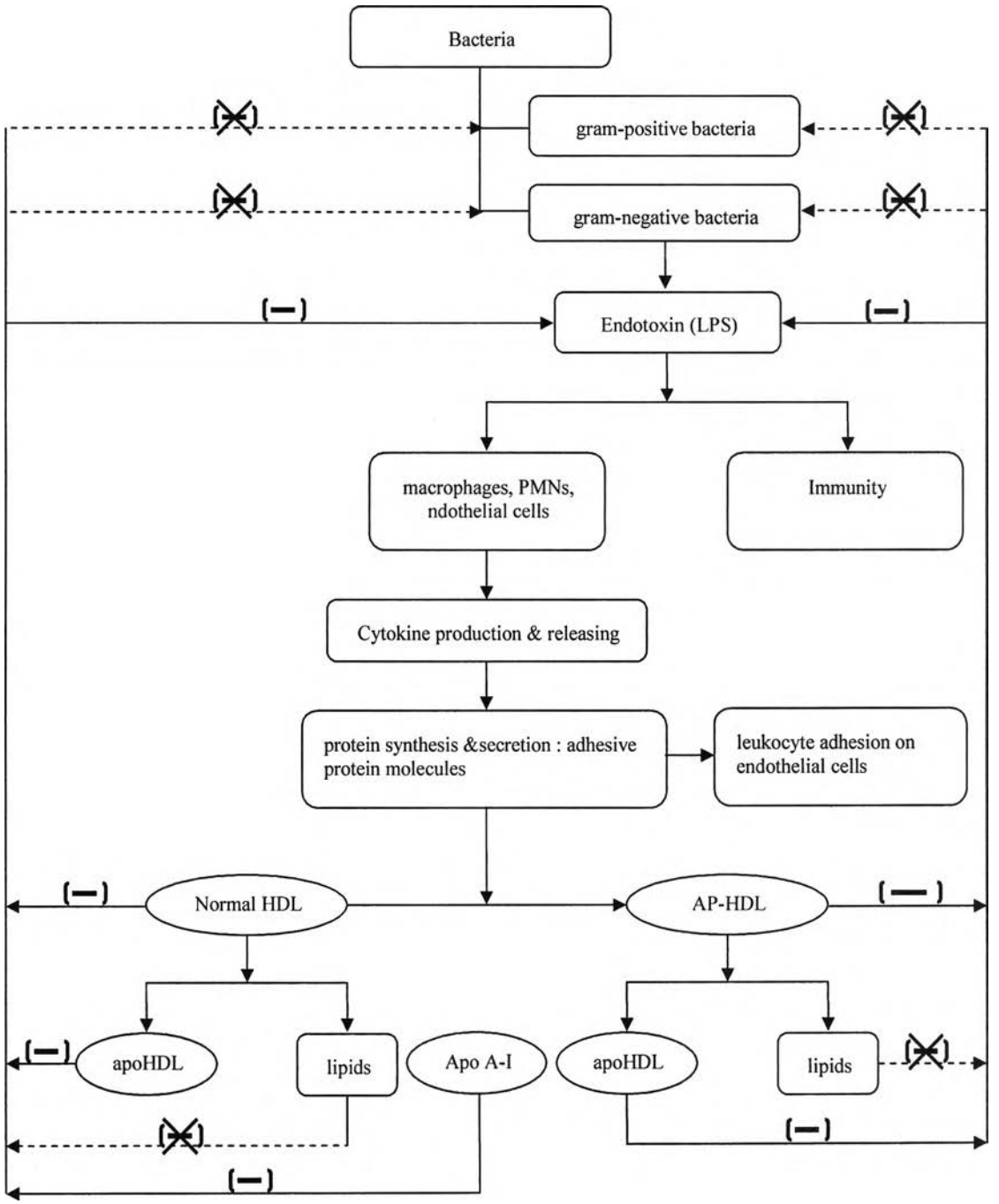
This study demonstrated that lipid-free apoHDL of normal HDL and AP-HDL could attenuate LPS-induced leukocyte adhesion (Figure 30). On the other hand, lipid component of HDL did not exert the inhibitory effect (Figure 31), suggesting that this inhibitory effect was due to the protein component of HDL.

On HDL, there are many proteins which may ameliorate the toxic effects of LPS. One of the protein candidates is lipopolysaccharide binding protein (LBP). LBP binds and transfers LPS not only to the receptor on the surface of macrophages and monocytes but also to HDL particles (Wurfel et al., 1994). During the APR, LBP level increases (Gabay et al., 1999, Hamannet al., 2005). *In vitro*, addition of low concentrations of LBP to macrophages enhanced LPS-induced TNF-α synthesis, but acute-phase concentrations of LBP were found to block this effect. In addition, high levels of LBP inhibited LPS-mediated cytokine release and reduced mortality rate *in vivo* (Wurfel et al., 1994). High levels of LBP during the APR might increase LPS transfer into AP-HDL, protecting against the toxic effect of LPS. Another protein candidate of HDL is phospholipid transfer protein (PLTP). PLTP mediates the exchange and transfer of phospholipids between lipoprotein particles (Tall et al., 1986). It was found that PLTP could also bind LPS and transfer to HDL, which resulted in LPS inactivation (Barlage et al., 2001). However, PLTP was unable to transfer LPS to the receptor on the surface of neutrophils and did not mediate responses of cells to LPS (Hailman et al., 1996). PLTP activity was increased in septic patients probably because of an increase in an active form of PLTP although the concentration of PLTP protein did not significantly increase (Barlage et al., 2001). Therefore, the increase in an active form of PLTP during the APR might cause AP-HDL to inhibit LPS-induced leukocyte adhesion on

endothelial cells more effectively than normal HDL. In addition, apolipoprotein E (apo E) is the one of protein components of HDL (Tuck, 2002), which could bind LPS (Van Oosten et al., 2001). Apo E was able to prevent the LPS-induced cytokine production and subsequent death in rodents and apo E-deficient mice demonstrated a significantly higher sensitivity toward LPS than control wild-type mice (Van Oosten et al., 2001). Moreover, apo E containing HDL was found to increase during septic condition (Barlage et al., 2001). The other protein candidate of HDL is apo A-I, the levels of which decrease during the APR (Hyka et al., 2001). Failure of LPS to induce fever was demonstrated following pre-incubation of LPS with apo A-I (Emancipator et al., 1992). Apo A-I inhibited the expression of adhesion proteins and chemokines induced by a periarterial collar in rabbits (Nicholls et al., 2005). In addition, purified human apo A-I was able to inhibit LPS-induced cytokine release (Flegel et al., 1993). Moreover, apo A-I was able to improve mortality rate of mice induced by LPS *in vivo* (Juan et al., 2004). Apo A-I mimetic peptide reduced LPS or lipid A-induced cytokine and adhesion molecules production *in vitro* (Gupta et al., 2005). These inhibitory effects of apo A-I were similar to our finding that normal human apo A-I was able to inhibit LPS-induced leukocyte adhesion on endothelial cells (Figure 32). This effect of apo A-I required incubation with LPS before administration into rats (Figure 34). In addition, apo A-I alone did not affect leukocyte adhesion on endothelial cells (Figure 33). It has been suggested that lipid-free apo A-I protein of HDL directly interacts with LPS due to amphiphatic structures of apo A-I, resulting in LPS inactivation. The most hydrophobic part of LPS which is the lipid A component could be bound and sheltered by the hydrophobic structure of apo A-I (Flegel et al., 1993). Another mechanism is that apo A-I may bind with LPS and cause conformational change of LPS that becomes inappropriate for the receptor (Flegel et al., 1993).

In these studies, the inhibitory effect of normal HDL and AP-HDL on LPS-induced leukocyte adhesion on endothelial cells required protein component but lipid component was not necessary. Apo A-I, the major structural protein of HDL, exerted similar effects as normal HDL. During the APR, apo A-I level decreased but other HDL-associated proteins were increased. Therefore, the higher inhibitory effect of AP-HDL on LPS-induced leukocyte adhesion on endothelial cells might be due to other HDL-associated proteins besides apo A-I. In addition, these studies indicated that the inhibitory effect of apo A-I on LPS-induced leukocyte adhesion on endothelial cells was due to interaction between apo A-I and LPS but not the direct effect of apo A-I on endothelial cells. However, normal HDL and AP-HDL were not able to suppress the growth of *S. epidermidis* and *E. coli* (Figure 35).

The roles of AP-HDL in antioxidative effects (Ehrenwald et al., 1994, Van Lenten et al., 1995) and reverse cholesterol transport are impaired (Tall, 1986, Cabana et al., 1989, Grunfeld et al., 1992, Khovidhunkit et al., 2000, Khovidhunkit et al., 2001, Barter, 2002). Therefore, these effects were also able to be potentially atherogenic. However, the role of AP-HDL in innate immunity to inhibit LPS-induced leukocyte adhesion on endothelial cells is improved during APR. AP-HDL might help the host to eliminate LPS that is the cause of atherogenic effect during APR. Beside the inhibitory effect of apo A-I on LPS-induced leukocyte adhesion on endothelial cells, other HDL-associated protein candidates, such as LBP, PLTP, might have the same inhibitory effect on LPS-induced leukocyte adhesion on endothelial cells. In addition, they might have synergistic effect on LPS-induced leukocyte adhesion on endothelial cells when they act together. These hypothesis need the further study.



(⊗) = can not inhibit

(—) = inhibit

(——) = higher inhibit

Figure 35 The proposed mechanisms of normal HDL and AP-HDL as an inhibitor of LPS