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Anti-inflammatory Effects of High-Density Lipoprotein via Regulation of Nitric Oxide Synthase Expression and Nf-κb Transcription in Activated Human Endothelial Cells

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ABSTRACT

Oxidation of low-density lipoprotein (LDL) and activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are critical for the inflammatory response for endothelial dysfunction. The objective of this study is to investigate the effects of various doses of HDL on: (a) LDL susceptibility to oxidation; (b) expression of eNOS; and (c) expression of NF-κB p50 and p65. Different concentrations of HDL were incubated in LDL. The reaction rates of LDL susceptibility to oxidation were obtained by kinetic modeling analysis. For determination of eNOS, NF-κB p50 and p65 expression, different HDL concentrations were incubated in lipo polysacharides (LPS)-stimulated human umbilical vein endothelial cell line for 16 hours. Protein was extracted and analysed by western blot and nuclear transcription factor, for example, Co-incubation of LDL with increasing HDL concentrations showed longer lag time and lower reaction rate in a dose-dependent manner compared to controls ($p<0.05$). The eNOS expression at higher HDL concentration was significantly increased when compared to controls ($p<0.05$). HDL significantly decreased the expression of NF-κB p65 but not that of NF-κB p50. HDL protects LDL from oxidation, up regulates eNOS expression and down regulates the expression of NF-κB p65. These in part contribute to the role of HDL in the prevention and retardation of atherogenesis and atherosclerosis-related complications.

Keywords: eNOS, NF-κB, endothelial cells, HDL, LDL oxidation, protein expression

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INTRODUCTION

Coronary heart disease (CHD) is the major cause of premature death in most populations including Malaysia. It is an important source of disability and substantially contributes to the escalating costs of health care. The commonest underlying pathology is atherosclerosis, which is a major cause of morbidity and mortality in the world (Roger, et al., 2012). It develops insidiously over time and is usually already in advance stage by the time the cardiovascular symptoms occur. Currently, a lot of evidence points at oxidative stress and inflammation as the precursor of atherogenesis (Hajjar & Gotto, 2013). High-density lipoprotein (HDL) plays an important role against the development of atherosclerosis and CHD. HDL is responsible in reverse cholesterol transport (Rothblat & Phillips, 2010), acts as an antioxidant (Kontush & Chapman, 2010), and regulates endothelial functions through its anti-inflammatory property (Säemannet et al., 2010), promotes nitric oxide synthase (eNOS) and activation (Mineo & Shaul, 2012b).

Oxidative stress and low-density lipoprotein (LDL) cholesterol oxidation are key factors in the pathogenesis of atherosclerosis. Peroxidation of LDL is known to be the first step in the development of atherosclerosis (Bekkering et al., 2014; Esterbauer, Striegl, Puhl, & Rotheneder, 1993). It is postulated that in oxidative stress, the reactive oxygen species (ROS) is directly involved in the various mechanisms of atherogenesis such as endothelial dysfunction, monocyte migration, smooth muscle proliferation and LDL oxidation (Singh, Mengi, Xu, Arneja, & Dhalla, 2002). Oxidised LDL is highly atherogenic, which leads to foam cell formation that further develops into atheromatous plaque and ultimately, the hardening of the artery (Yu, Fu, Zhang, Yin, & Tang, 2013).

Endothelial cells maintain basal vascular tone and actively regulate vascular reactivity in physiological and pathological conditions. They respond to mechanical forces and a variety of neuro humoral mediators. Nitric oxide (NO) is one of the most important vasoactive substances released by the endothelium, which is not only used as a vasodilator (Förstermann & Sessa, 2012), but also inhibits cellular inflammation (Kobayashi, 2010). In non-pathological conditions, NO is synthesised by enzymatic conversion of L-arginine, in the presence of molecular oxygen, by endothelial eNOS which is expressed constitutively in endothelial cells (Tousoulis, Kampoli, Tentolouris, & Stefanadis, 2012). Endotoxins, such as tumor lipopolysacharides (LPS) play a critical role in propagation of endothelial dysfunction by down regulating the expression of eNOS, resulting in decreased bio availability of nitric oxide (Wilson et al., 2000).

The ubiquitously expressed nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor that regulates many cellular processes including cell proliferation, survival, adhesion, and immunity. NF-κB is activated in the presence of stimuli such as in the event of oxidative stress, by cytokines or by bacterial LPS (Gupta, Sundaram, Reuter, & Aggarwal, 2010). Activated NF-κB is an important regulator for inflammatory mediators such as interleukin-1 and tumor necrosis factor (TNF) (Eda Shimada, Beidler, & Monahan, 2011). NF-κB is retained and remains inactivated in the cytosol through sequestration by nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (I-κB) proteins. NF-κB is activated by I-κB kinase (IKK) which degrades I-κB through phosphorylation. A large

variety of stimuli potentially induce activation of NF-κB which, in concert with transcriptional coactivators, lead to the induction of a multitude of target genes (Yamamoto & Gaynor, 2001).

The most prevalent active form of NF-κB is a heterodimer composed of subunits p50 and p65. Deficiency of p65 is lethal due to its association with developmental abnormalities (Novack, 2011). Although p50 knockout mice can develop normally, B-cell proliferation is disrupted and antibody production is compromised (Franzoso et al., 1997). However, over-activation of NF-κB is associated with various disorders. In auto-immune state, sustained activation of NF-κB causes inflammatory myopathies (Creus, De Paepe, & De Bleecker, 2009), while in the events of oxidative stress caused by hyperglycaemia or hypercholesterolaemia, sustained activation of NF-κB is associated with atherosclerosis (Real et al., 2010; Vanessa Fiorentino, Prioletta, Zuo, & Folli, 2013).

To date, the mechanism HDL's antiatherosclerotic effect is still considered complex and its role as an antioxidant and anti-inflammatory agent in terms of attenuating atherosclerosis is still not fully understood. The role of HDL in anti antherosclerosis was made even controversial when it was found that the HDL in people with coronary artery disease (CAD) does not upregulate NOS as in healthy people due to activation of endothelial protein kinase Cβ II by the former that disrupts the eNOS activation pathway (Besler et al., 2011). However, since a lot of evidence has also indicated that HDL is inversely correlated with the incidence of CHD (Feig et al., 2011; Mackey et al., 2012; Vergeer, Holleboom, Kastelein, & Kuivenhoven, 2010), HDL may not simply exert its athero protective effect by a single pathway. Therefore, the objective of this study is to investigate whether the HDL can downregulate the expression of NF-κB in human endothelial cells, which may in turn upregulate the expression of eNOS. The ability of HDL to reduce the susceptibility of LDL to oxidation is also investigated.

MATERIALS AND METHODS

Plasma Source

Approval from UiTM Board of Ethics Committee was obtained prior to the commencement of the study [reference number: 600-RMI (5/1/6/01)]. Pooled plasma was obtained from 20 healthy individuals from Faculty of Medicine, UiTM.

Lipoprotein Isolation

Plasma was adjusted to a density of 1.21 g/mL by adding 1.1 mL of pooled human plasma to 0.3270 g of potassium bromide and was mixed gently by vortex mixer. A discontinuous density gradient was made by overlaying the plasma solution with 2.2 mL of saline containing 0.1% ethylenediaminetetraacetic acid in ultracentrifuge tubes (d=1.006 g/mL). The ultracentrifuge tubes were sealed and centrifuged at 100,000 rpm at 4°C for 40 minutes using an ultracentrifuge (Optima TLX, Beckman Coulter, Palo Alto, CA). HDL fraction was placed in a nitrocellulose bag and was dialysed at 4°C for 24 hours. The total protein (TP) concentration was determined using a bio analyser (Cobas Integra 400 plus, Roche Diagnostics Limited, Rotkreuz, Switzerland).

The Effects of HDL on LDL Susceptibility to Oxidation

Preparation of LDL and conjugated dienes formation was determined as described by Gieseg and Esterbauer (1994). Freshly prepared LDL (50 µg/mL) was incubated with different concentrations of HDL (20, 40, 60 and 80 µg/mL) which covered the lower and higher concentration than that of the LDL. The mixtures were oxidised with 0.5µM copper sulphate ($CuSO_4$) at 37°C for three hours. The conjugated dienes formation detected by the spectrophotometer (234 mm) represents the degree of lipid peroxidation in the sample, where the more conjugated dienes is detected, the more the LDL is susceptible to oxidation (Moore & Roberts, 1998). Lag time (min), which is the time interval between the addition of $CuSO_4$ and the beginning of the oxidation, represents how long the LDL is protected by the HDL before it is oxidised. The reaction rate (absorbance/minute) is the maximal rate of LDL oxidation detected in the kinetic curve and maximum absorbance of lipid peroxidation was obtained by kinetic modelling analysis (Esterbauer, Wäg, & Puhl, 1989).

HUVEC Isolation, Culture and Incubation Conditions

Human umbilical vein endothelial cell line (HUVEC) was purchased from Cambrex Bio Science Walkersville, USA. HUVEC (1 x 10^6 cells/mL) was cultured in 75 cm2 flask in medium 199 containing 20% foetal calf serum, 10,000 U/mL penicillin/streptomycin, 100 mg/L heparin, 3 mmol/l L-glutamine and 15 mg/mL endothelial cell growth supplement. HUVEC was stimulated with LPS (1µg/mL) to induce inflammatory response and different concentrations of HDL [20-80 µg/mL for LDL oxidation susceptibility study; 20-120 mg/dL for eNOS, NF-κB p50 and p65 expression studies based on the optimum HDL concentration that supress cell apoptosis (Nofer et al., 2001) were added to the separated HUVEC cultures. Each HUVEC cultures with different HDL concentrations was prepared in triplicates. The HUVEC cultures were incubated in a humidified incubator (37°C, 5% CO_2) for 16 hours.

The Effects of HDL on eNOS Expression

Stimulated HUVEC was homogenised in celllysis buffer containing 10 mmol/L Tris (pH 7.4), 1 mmol/L sodium ortho-vanadate and 1% (w/v) sodium dodecyl sulfate. Protein concentrations in the lysates were measured using the bioanalyser. Proteins (50 µg) were analysed by western blot method according to the standard procedure. Monoclonal antibodies against human eNOS, were used as primary antibodies and horseradish peroxidise (HRP) goat anti-mouse antibody was used as secondary antibody. The bands were visualised and quantitated using the BioRad Gel system with Quantity One Quantitation Software (BioRad Laboratories, Hercules, CA, USA).

The Effects of HDL on NF-κB p50 and p65 Expression

HUVEC was extracted using a nuclear extraction kit (Cayman Chemical, Ann Arbor, MI, USA). The cells were collected on ice-cold phosphate-buffered saline in the presence of phosphatase inhibitors. The pelleted cells were then re-suspended in ice-cold hypotonic buffer, followed by the addition of10% Nonidet-p40 and were centrifuged. Protein concentrations in the lysates (50 µg) were measured with NF-κB (human p50 and p65) Transcription Factor

Assay kit (Cayman Chemical, Ann Arbor, MI, USA). The expression of NF-κB was detected using the bioanalyser. NF-κB was detected by addition of specific primary antibody directed against NF-κB. A secondary antibody conjugated to HRP was added to provide a sensitive colorimetric readout at 450 nm.

Statistical Analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS) version 22.0. Data are presented as mean ± SD. The effects of different concentrations of HDL were compared by analysis of variance. Comparison between two different groups was analysed using independent T-test. Significant level was set at $p<0.05$.

RESULTS

The Effects of HDL on LDL Susceptibility to Oxidation

Co-incubations of LDL with HDL increased the mean lag time of diene formation and produced a slower rate of oxidation across all HDL concentration (Figure 1). On the contrary, the amounts of conjugated dienes generated by LDL during oxidation were significantly increased when co-incubated with 40 µg/mL of HDL and above.

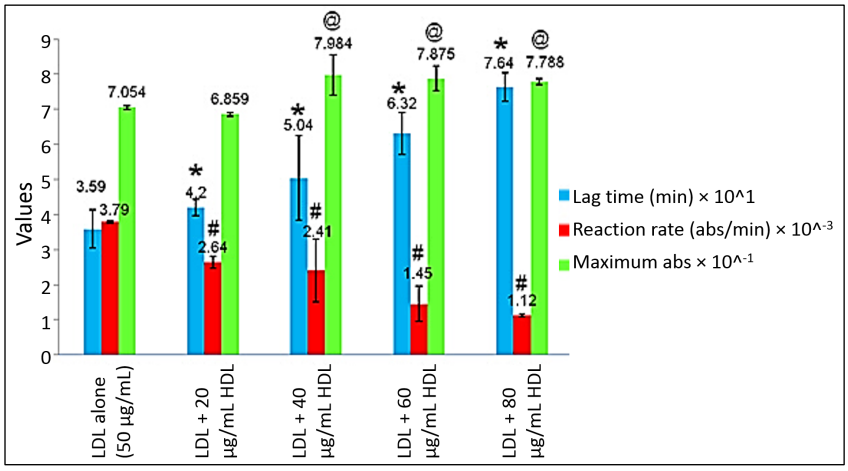

Figure 1. Mean lag time, oxidation reaction rate and amount of conjugated dienes formation by oxidised LDL (in the unit of maximum abs) with the presence of different concentrations of HDL

Mean lag times was gradually increased, while oxidation reaction rates were gradually decreased respective to the increase of co-incubated HDL concentration. *$p<0.05$ compared to LDL alone (Lag time), #$p<0.05$ compared to LDL alone (Reaction Rate), @$p<0.05$ compared to LDL alone (Maximum abs).

The Effects of HDL on Expression of eNOS

The eNOS was expressed as the 133 kDa band in the western blot shown in Figure 2. The changes in eNOS were determined after densitometric measurement of the western blots (Figure 3). Without addition of HDL or LPS, the endothelial cells in negative control expressed eNOS at normal rate. LPS successfully reduced the expression of eNOS by endothelial cells, demonstrated by the reduced band intensity in the positive control. HDL not only successfully mitigates the effect of LPS, but increased the expression of eNOS beyond its normal expression rate (negative control). At higher concentrations of HDL, (100 and 120 mg/dL) the expression of eNOS were significantly increased. At lower HDL concentrations (20-80 mg/dL), however, eNOS inductions were weaker and not significantly different when compared with the positive controls.

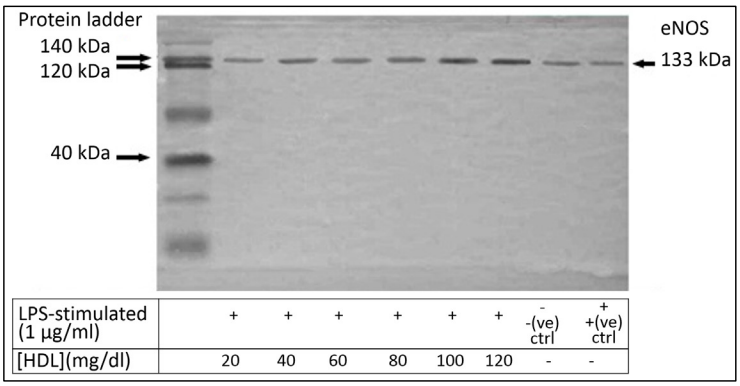

Figure 2. Western blot image of eNOS protein expression in HUVEC after stimulated with LPS and incubated with different concentrations of HDL for 16 hours

The band intensities were gradually increased from 20 to 120 mg/dL of HDL co-incubation. HUVEC incubated with LPS alone was used as positive control. Unstimulated HUVEC samples without HDL were used as negative controls.

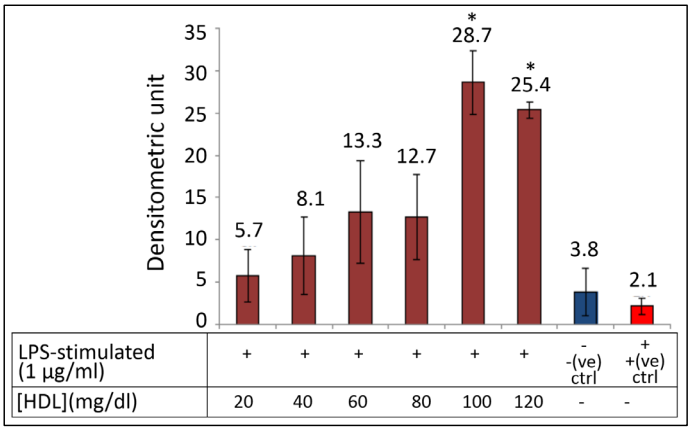

Figure 3. Western blot's quantitative result of eNOS protein expression in HUVEC after stimulated with LPS and incubated with different concentrations of HDL for 16 hours

HUVEC sample incubated with LPS alone was used as positive control. *p<0.05 when compared with positive control. Unstimulated HUVEC samples without HDL were used as negative controls.

The Effects of HDL on Expression of NF-κB in LPS-Stimulated Endothelial Cell

Unlike the eNOS expression experiment, the expression of NF-κBp50 and p65 have to be down regulated in order to demonstrate the protective effect of HDL. After the LPS stimulation, the expression of NF-κB p50 without co-incubation with HDL (positive control) was increased by 23% when compared with negative control (Figure 4). The expression of NF-κB p50 after treatment with different concentrations of HDL, however, was not significantly different when compared with the positive control. On the other hand, the expressions of NF-κB p65 significantly decreased across all the HDL concentrations when compared with the positive control (Figure 5).

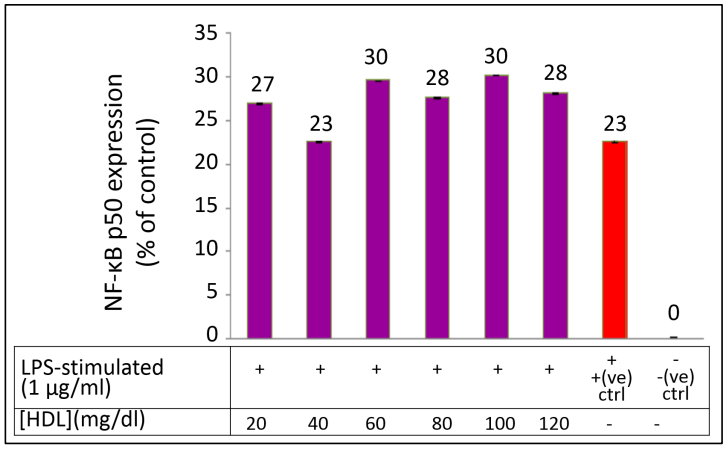

Figure 4. The effects of different concentrations of HDL on expression of NF-κB p50

The expressions NF-κB p50 were of not significantly different than that of positive control regardless of whether the HDL concentration was co-incubated with the LDL. HUVEC sample incubated with LPS alone was used as the positive control. Unstimulated HUVEC sample without HDL was used as negative control. Data are presented as % changes of NF-κB versus unstimulated HUVEC samples.

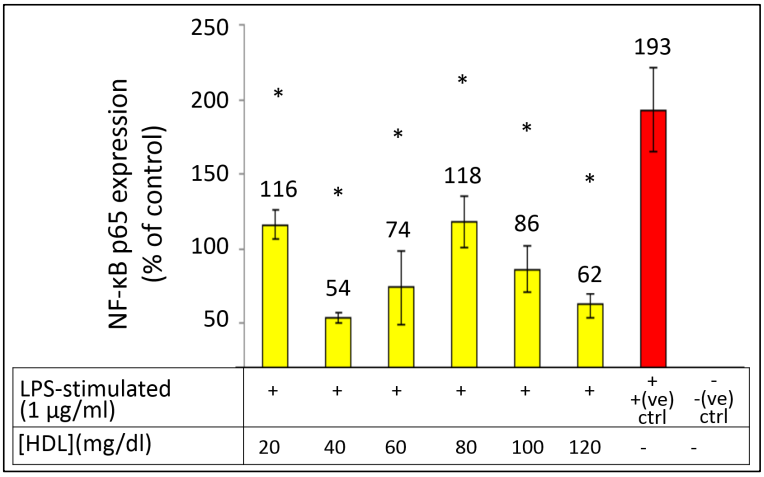

Figure 5. The effects of different concentration of HDL on expression of NF-kB p65

The expressions of NF-kB p65 were significantly downregulated in all LDL co-incubated with HDL. HUVEC sample incubated with LPS alone was used as positive control. Unstimulated HUVEC sample without HDL was used as negative control. Data are presented as % changes of NF-kB versus unstimulated HUVEC samples. *$p<0.05$ compared to positive controls.

DISCUSSION

This study demonstrated that HDL reduces LDL susceptibility to oxidation, evidenced by the increased oxidation lag time and reduced oxidation reaction rate, in proportion with the concentration of HDL co-incubated with the LDL. However, the maximum absorbance of conjugated dienes that represent the amount of oxidised LDL, are increased when co-incubated with HDL 40 µg/mL onwards. Thus, the optimum concentration of HDL to inhibit LDL oxidation appears to be only 20 µg/mL. These findings contradict with the previously elucidated reports on anti oxidative property of HDL. However, an *ex vivo* study conducted by Solakiviet et al. (2005) was in agreement with our finding, which demonstrated that co-incubation of HDL in LDL samples not only increase the maximum dienes concentration, but also decrease the lag time and increase the rate of reaction of dienes formation (Solakiviet al., 2005). They hypothesised that HDL is even more susceptible to oxidation than LDL when a high concentration of Cu^{2+} (1.65 µM as used in their experiment) is used for induction of oxidation, suggesting that HDL only exerts its protective property in mildly oxidative environment, but not so in severe oxidative stress, probably due to the deactivation of serum paraoxonase 1 enzyme, which is usually associated with HDL to protect in from oxidation (Aviram et al., 1999). Nevertheless, the authors did not deny the contradicting findings of their study against several other studies (Bowry, Stanley, & Stocker, 1992; Ohmura et al., 1999; Schnitzer, Pinchuk, Fainaru, Schafer, & Lichtenberg, 1995; Suzukawa, Ishikawa, Yoshida, & Nakamura, 1995), were probably contributed by the non-standardised concentration of Cu^{2+} used in the studies and other potential individual intrinsic factors, such as size of the lipoproteins (Chait, Brazg, Tribble, & Krauss, 1993; Kontush, Chantepie, & Chapman, 2003) and blood glucose

level. Larger particle size of HDL is often associated with lower risk of cardiovascular disease (Kontush, 2015). Individuals with increased body mass index tend to have higher level of small particle size HDL (Williams et al., 1993). The standardisation of these purported intrinsic factors were not taken into account in this study, therefore might be responsible for producing this contradictory result. The increase of conjugated dienes formation at HDL 40 µg/mL and above also can be explained by the enhanced oxidation of HDL itself at higher concentration, particularly in a highly oxidative environment. Nevertheless, the findings of this study supports HDL as an inhibitor to LDL susceptibility to oxidation in terms of prolonged lag phase and diminished reaction rate.

The protective effect of HDL is contributed by multiple pathways, including moderating endotheliumapoptosis, proliferation and migration, acts as anti-inflammatory agent and inhibits platelet aggregation (Mineo & Shaul, 2012b). In its antioxidative role, HDL re-stimulates eNOS, which has been repressed by oxidised LDL, through binding with scavenger receptor–B1 on cell membrane (Garcia-Cardena, Oh, Liu, Schnitzer, & Sessa, 1996; Stein & Stein, 1999). In this study, HDL not only mitigated the downregulation of eNOS caused by LPS, but also increased the eNOS expression beyond its normal rate, which was represented by the negative control that was not even suppressed by LPS in the beginning. However, the stimulation of eNOS by HDL is concentration-dependent. In this study, lower concentration of HDL (20 to 80 mg/dL), were not sufficient to exert significant increase in eNOS expression, even though the trend of gradual increase was still observable. The significant benefit effect of HDL was only exerted in high concentration (100 and 120 mg/dL).

This non-convincing finding, however, does not discredit the potential benefits of HDL. Pathologically increased activation of NF-κB suppresses the eNOS expression (Lee, et al., 2014), therefore exacerbates the atherogenesis. HDL mitigates the downregulation of eNOS by suppressing the NF-κB. The ability of HDL to suppress the activation of the readily expressed NF-κB is well-documented, primarily studied by using electrophoretic mobility shift assay (Da Silveira Cruz□Machado, et al., 2010; Sanz, et al., 2010). The HDL prevents NF-κB activation by neutralising the toxic effect of LPS (Wang, et al., 2008).

However, the effect of HDL on the expression of NF-κB is not well-studied. A study conducted by Kastenbauer and Ziegler-Heitbrock (1999) demonstrated that the expression of both NF-κB p50 and p65 sub units did not show similar response to LPS exposure, where the expression of p50 increased three-folds when compared with that of p65 after two to three days of LPS exposure. In this study, the expression of both NF-κB p50 and p65 were upregulated in positive controls (Zhang et al., 2008). However, the enhanced expression of NF-κB p50 were not as strong as that of p65. Co-incubation of HDL in NF-κB p50 samples did not significantly reduce its expression either, probably because it was already very under expressed to be further suppressed by HDL. The different findings were probably due to the shorter sample incubation time used in this study (16 hours), compared to that by Kastenbauer and Ziegler (two to three days). The unsuppressed NF-κB p50 are, in fact, beneficial for the reduction of inflammatory mediator TNF. The increased amount of NF-κB p50 forms p50 homodimers which competitively binds to the target gene with p50/p65 heterodimers (Kastenbauer & Ziegler-Heitbrock, 1999). The p50 does not activate the expression of the targeted gene because the protein terminal of p50 does not contain the transcription activation domain such in that of

p65, which is required for the gene activation (Ghosh & Hayden, 2012). The protective effect of HDL is demonstrated in this study as it does not downregulate the expression of NF-κB p50, but significantly downregulates the expression of NF-κB p65.

CONCLUSION

Overall, HDL protects the LDL from oxidation, but it may enhance LDL oxidation in highly oxidative environment. High concentration of HDL increases eNOS expression and inhibits NF-κB expression, especially the p65 subunit, even in low concentrations. Hence, HDL has antioxidant and anti-inflammatory properties, not only by prevention oxidation, but also by its roles in the eNOS and NF-κB pathways. This may in part attribute to its role in reverse cholesterol transport. However, the mechanism of how HDL regulates the expression of NF-κBis is still largely unknown. Future studies are warranted in addressing this issue.

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