Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, Dalian, China

Naringin regulates cholesterol homeostasis and inhibits inflammation via modulating NF-κB and ERK signaling pathways *in vitro*

JING LIANG, CHANGYUAN WANG, JINYONG PENG, WENSHUANG LI, YUE JIN, QI LIU, QIANG MENG, KEXIN LIU, HUIJUN SUN

Received April 24, 2015, accepted May 25, 2015

Huijun Sun, Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, 9 West Section, Lvshun South Road, Lvshunkou District, Dalian 116044, China
sunhuijun@dlmedu.edu.cn

Pharmazie 71: 101–108 (2016) *doi: 10.1691/ph.2016.5629*

The main purpose of this study was to examine if naringin contributed to the regulation of cholesterol homeostasis and inflammatory cytokine expressions in cholesterol and 25-OH-cholesterol-treated HepG2 cells and TNF-α-treated HUVECs. The gene and protein expressions related to cholesterol homeostasis and inflammation were determined by quantitative real-time reverse transcription-polymerase chain reaction and Western blotting. We obtained the following results: (1) A concentration-dependent increase of LDLR and CYP7A1 expressions was observed, through activating expressions of SREBP2 and PPARγ in HepG2 cells after exposure to naringin; (2) EL gene and protein expressions in HUVECs were inhibited by naringin; (3) the expressions of inflammatory factors such as CRP, TNF-α, ICAM-1 and VCAM-1 in HepG2 cells, ICAM-1 and VCAM-1 in HUVECs restrained by naringin were confirmed; (4) NF-κB and ERK1/2 activities were quenched by naringin. In summary, naringin might not only effectively reduce cholesterol levels by stimulating cholesterol metabolism but also inhibit inflammatory response through reducing inflammatory cytokine expressions. The effects of naringin were achieved *via* modulating NF-κB and ERK signaling pathways.

1. Introduction

Lipid accumulation is the major pathogenic event of metabolic disorders especially atherosclerosis (Ren and Ning 2014). As a signaling molecule lipid plays a major regulatory role in the gene expressions of cytokines involved in inflammatory responses which may trigger initial events that can lead to atherosclerotic cardiovascular disease.

Elevated plasma low density lipoprotein (LDL) cholesterol (LDL-C) levels are associated with cardiovascular diseases (Norata 2014). In most metabolic states, regulation of circulating LDL-C levels is controlled in large part by the rate of liver uptake and clearance of LDL-C particles by hepatocyte cell surface low density lipoprotein receptor (LDLR) (Melone et al. 2012). The expression of LDLR is regulated by sterol regulatory element-binding protein-2 (SREBP2) at the transcriptional level (Horton et al. 2002) and the cardinal role for SREBP2 in maintaining and/or altering cholesterol homeostasis is compellingly documented in genetic experiments (Weber et al. 2004).

In the liver, free cholesterol is further metabolized into bile acids for excretion by the action of the rate-limiting enzyme, cholesterol 7α-hydroxylase (CYP7A1) to maintain cholesterol homeostasis (Defesche 2004). Peroxisome proliferator-activated receptor γ(PPARγ) pertains to the PPAR family of ligand-activated transcription factors (Brown and Plutzky 2007). PPARγ plays an important role in different biological processes and has been shown to be atheroprotective because of inducing CYP7A1 expression and LDLR expression/function to enhance LDL cholesterol metabolism (Duan et al. 2012). Previous studies indicated that the induction of LDLR expression by PPARγ was sterol regulatory element-dependent because PPARγ enhanced SREBP2 processing (Duan et al. 2012). Several studies have shown that extracellular signal regulated kinases (ERK) pathway plays essential roles in adipogenesis and the ERK pathway has been shown to regulate negatively PPARγ (Duan et al. 2012).

A low level of high density lipoprotein (HDL) is linked to risk factors like atherosclerosis and hyperlipidemia (Di Angelantonio et al. 2009; Vega and Grundy 1996). Recently, endothelial lipase (EL), a member of the triglyceride lipase gene family, has been considered as a key enzyme that modulates HDL metabolism (Hirata et al. 1999; Jaye et al. 1999). Overexpression of EL *in vivo* resulted in reduced plasma HDL levels and similar results were subsequently reported in EL transgenic mice (Ishida et al. 2003; Maugeais et al. 2003).

It is also well established that inflammation plays a key role in all stages of the atherosclerotic process, from lesion initiation to progression and ultimately to plaque rupture and thrombosis (Libby et al. 2002; Lusis 2000). Inflammatory cytokines like C reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α) play crucial roles in the process of atherosclerosis, because they are closely related to activation of monocytes/macrophages which are the major sources of vasoactive substances, coagulation factors, and matrix degrading enzymes (E et al. 2010). TNF-α up-regulates EL expression which is also correlated with the expressions of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Sasaki et al. 2005; Wu et al. 2010). And the expressions of the inflamma-

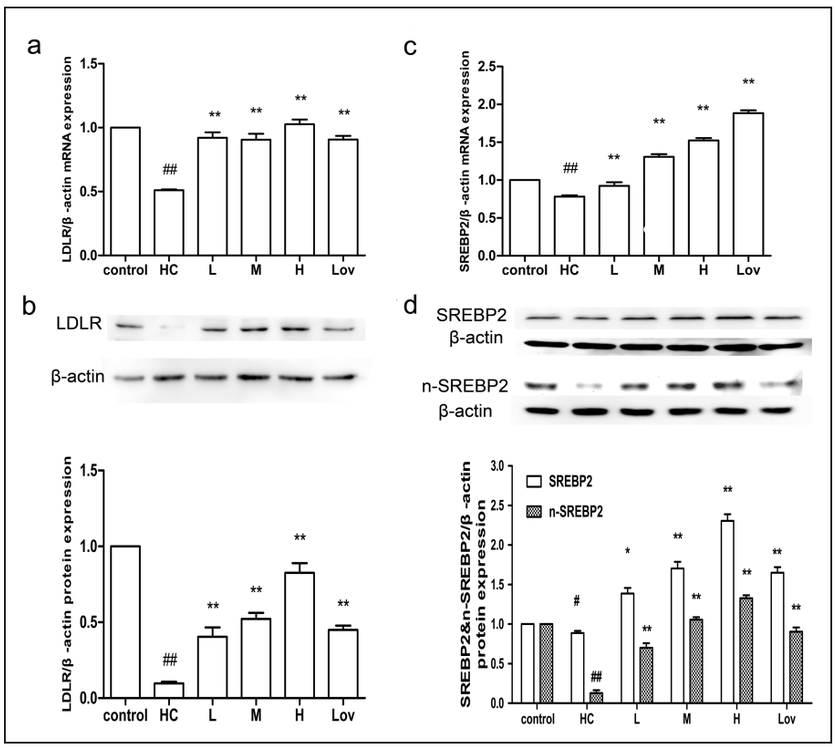

Fig. 1: Effects of naringin on LDLR and SREBP2 expression in HepG2 cells. HepG2 cells were incubated in medium containing cholesterol (10 μg/mL) and 25-OH-cholesterol (1 μg/mL) for 24 h, and then were treated with naringin (25, 50, 100 μg/mL) or lovastatin (1 μM) for 24 h. (a) The mRNA expression of LDLR in HepG2 cells determined by qPCR. (b) The protein expression of LDLR in HepG2 cells determined by Western blotting. (c) The mRNA expression of SREBP2 in HepG2 cells determined by qPCR. (d) The protein expressions of total SREBP2 and nuclear SREBP2 in HepG2 cells determined by Western blotting. Each bar represents the mean ± SEM of triplicate determinations. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to control; $^{*}p<0.05$, $^{**}p<0.01$ compared to HC group.

tory genes of ICAM-1 and VCAM-1 are tightly regulated by activation of NF-κB, revealing that transcriptional activation of nuclear factor-κB (NF-κB) is a critical step in the pathogenesis of atherosclerosis (Kim et al. 2013).

Naringin (4,5,7-trihydroxy-flavonone-7-rhamnoglucoside) is an active flavonoid isolated from citrus fruit extracts. Naringin is known to exert multiple pharmacological properties, including anti-oxidation, anti-inflammation and anti-hypercholesterolaemia (Jain and Parmar 2011; Jeon et al. 2004). However, molecular mechanisms by which naringin influences cholesterol homeostasis and supresses inflammation remain unknown. Statins have a cholesterol-lowing effect in addition to their substantial amount of pleiotropic effects against hypercholesterolaemia and inflammation (Davidson et al. 2003; Wu et al. 2015). Therefore, we use lovastatin as a positive control to investigate the effect of naringin supplementation on both hypercholesterolaemia and inflammatory response *in vitro*, to uncover the responsible mechanisms.

2. Investigations and results

2.1. Effects of naringin on LDLR expression in HepG2 hepatocytes

First, significant reduction of LDLR mRNA and protein expressions were observed in cells treated by cholesterol and 25-OH-cholesterol, compared with the control group (Fig. 1a and 1b, $p<0.01$). Compared with the HC group, naringin treatment significantly up-regulated LDLR expression ($p<0.01$). LDLR mRNA and protein expressions were also increased by lovastatin treatment, the positive control.

2.2. Effects of naringin on SREBP2 expression in HepG2 hepatocytes

Then the effect of naringin on the expression of SREBP2, the major transcription factor regulating the expression of LDLR, was examined. As shown in Fig. 1c, HC decreased the expressions of SREBP2 mRNA, but naringin markedly improved gene expression of SREBP2 in a concentration-dependent manner in cells treated by HC. Naringin increased the nuclear SREBP2 expression more significantly rather than total SREBP2 protein expression in HC-treated HepG2 cells (Fig. 1d). As a positive control drug, lovastatin had the same effect.

2.3. Effects of naringin on CYP7A1 expression in HepG2 hepatocytes

Thereafter the effect of naringin on the expression of CYP7A1, the rate-limiting enzyme in maintaining cholesterol homeostasis, was also examined. As show in Fig. 2a and 2b, HC decreased the expressions of CYP7A1 mRNA and protein. However, naringin treatment was associated with a concentration-dependent increase in the expressions of CYP7A1 mRNA and protein.

2.4. Effects of naringin on PPARγ expression and ERK1/2 activation in HepG2 hepatocytes

Because PPARγ plays a major regulatory role in regulating the expression of genes involved in cholesterol metabolism (Li et al. 2013), we examined whether its expression would be modulated by naringin in HepG2 cells. As shown in the Western blot-

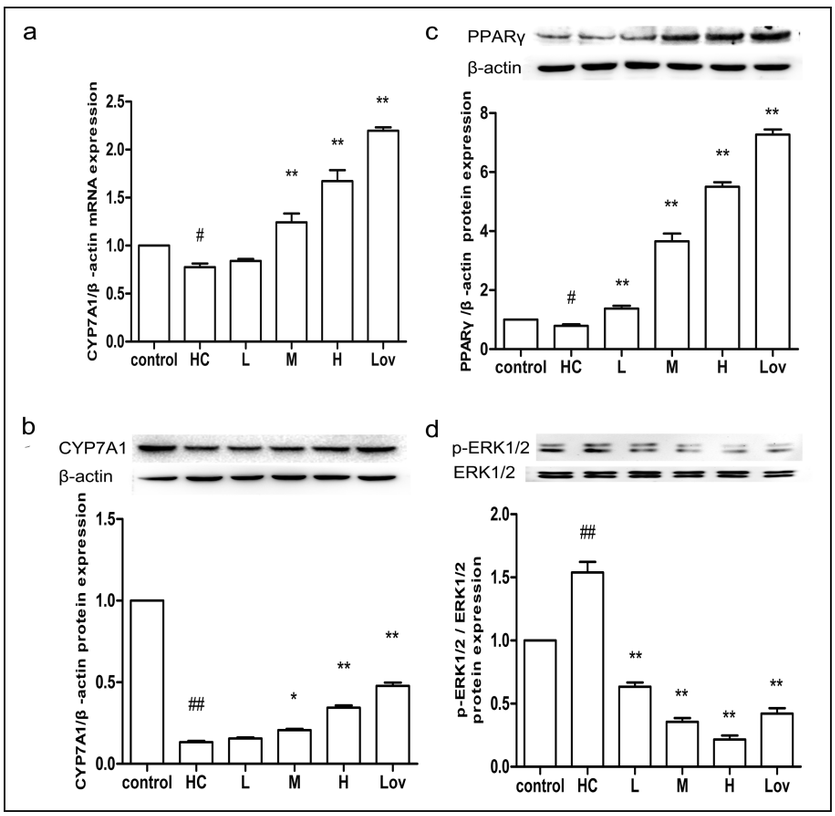

Fig. 2: Effects of naringin on CYP7A1 expression, PPARγ expression, and ERK1/2 activation in HepG2 cells. HepG2 cells were incubated in medium containing cholesterol (10 μg/mL) and 25-OH-cholesterol (1 μg/mL) for 24 h, and then were treated with naringin (25, 50, 100 μg/mL) or lovastatin (1 μM) for 24 h. (a) The mRNA expression of CYP7A1 in HepG2 cells determined by qPCR. (b) The protein expression of CYP7A1 in HepG2 cells determined by Western blotting. (c) The protein expression of PPARγ in HepG2 cells determined by Western blotting. (d) The protein expressions of total ERK1/2 and p-ERK1/2 in HepG2 cells determined by Western blotting. Each bar represents the mean ± SEM of triplicate determinations. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to control; $^{*}p<0.05$, $^{**}p<0.01$ compared to HC group.

ting analysis results (Fig. 2c), protein expression of PPARγ was inhibited by HC, but this inhibition was substantially reversed by the treatment with naringin. Lovastatin as a positive control also increased the expression of PPARγ. ERK1/2 is the upstream of PPARγ (Kwak et al. 2012), thus effects of naringin on ERK1/2 activation were then determined. The relative level of p-ERK1/2 in HC-treated HepG2 cells, determined as the ratio of p-ERK1/2 to ERK1/2, was higher (Fig. 2d, $p<0.01$) compared to control group. In HepG2 cells treated with naringin or lovastatin, ERK1/2 phosphorylation was significantly lower ($p<0.01$) than in HepG2 cells exposed to cholesterol and 25-OH-cholesterol.

2.5. Effects of naringin on inflammatory cytokines in HepG2 cells

In order to observe the role of naringin regulating inflammatory cytokines in HepG2 cells, ICAM-1, VCAM-1, TNF-α and CRP expressions were detected. As shown in Fig. 3, expressions of ICAM-1, VCAM-1, TNF-α and CRP were increased by the treatment with cholesterol and 25-OH-cholesterol. These inductions were substantially reduced by the treatment with naringin. Lovastatin as a positive control also reduced the expressions of ICAM-1, VCAM-1, TNF-α and CRP.

2.6. Effects of naringin on EL expression in HUVECs

It is well known that EL plays an important role in lipid metabolism and inflammatory response (Kivelä et al. 2010). Moreover EL was adjusted by proinflammatory cytokines (Yasuda et al. 2007). Thus we evaluated the regulating effects of naringin on mRNA and protein levels of EL in TNF-α-induced HUVECs. As shown in Fig. 4a, b, treatment with naringin caused a significant reduction in EL mRNA and protein over-expression induced by TNF-α, and as a positive control, lovastatin treatment also decreased mRNA and protein expressions of EL.

2.7. Effects of naringin on inflammatory cytokines in HUVECs

Then we examined the effects of naringin on expressions of inflammatory cytokines ICAM-1 and VCAM-1 in TNF-α-treated HUVECs (Fig. 4c-f). The expressions of ICAM-1and VCAM-1 were obviously promoted in TNF-α treated group compared with the control group ($p<0.01$), but naringin or lovastatin inhibited the effect of TNF-α significantly.

2.8. Effects of naringin on NF-kB activation of HepG2 cells and HUVECs

Finally, to demonstrate the effects of naringin on regulating NF-κB pathway, we examined the cytoplasmic I-κB level and nuclear NF-κB p65 level by western blotting analysis. The data showed that treatment with naringin was able to up-regulate the protein level of cytoplasmic I-κBα and down-regulate the level of nuclear NF-κB p65 in both HUVECs (Fig. 5a, b) and HepG2 (Fig. 5c, d) cells in a concentration-dependent manner. As a positive control, lovastatin treatment also inhibited NF-κB activation.

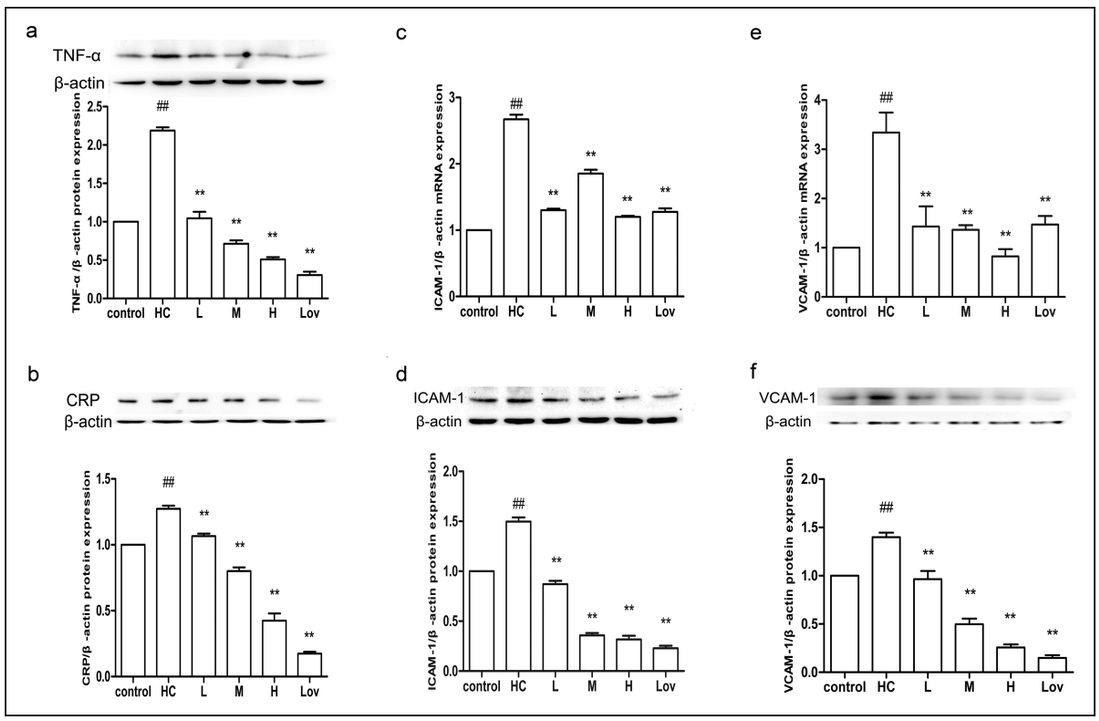

Fig. 3: Effects of naringin on inflammatory cytokines in HepG2 cells. HepG2 cells were incubated in medium containing cholesterol (10 μg/mL) and 25-OH-cholesterol (1 μg/mL) for 24 h, and then were treated with naringin (25, 50, 100 μg/mL) or lovastatin (1 μM) for 24 h. (a) The protein expression of TNF-α in HepG2 cells determined by Western blotting. (b) The protein expression of CRP in HepG2 cells determined by Western blotting. (c) The mRNA expression of ICAM-1 in HepG2 cells determined by qPCR. (d) The protein expression of ICAM-1 in HepG2 cells was determined by Western blotting. (e) The mRNA expression of VCAM-1 in HepG2 cells determined by qPCR. (f) The protein expressions of VCAM-1 in HepG2 cells determined by Western blotting. Each bar represents the mean ± SEM of triplicate determinations. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to HC group.

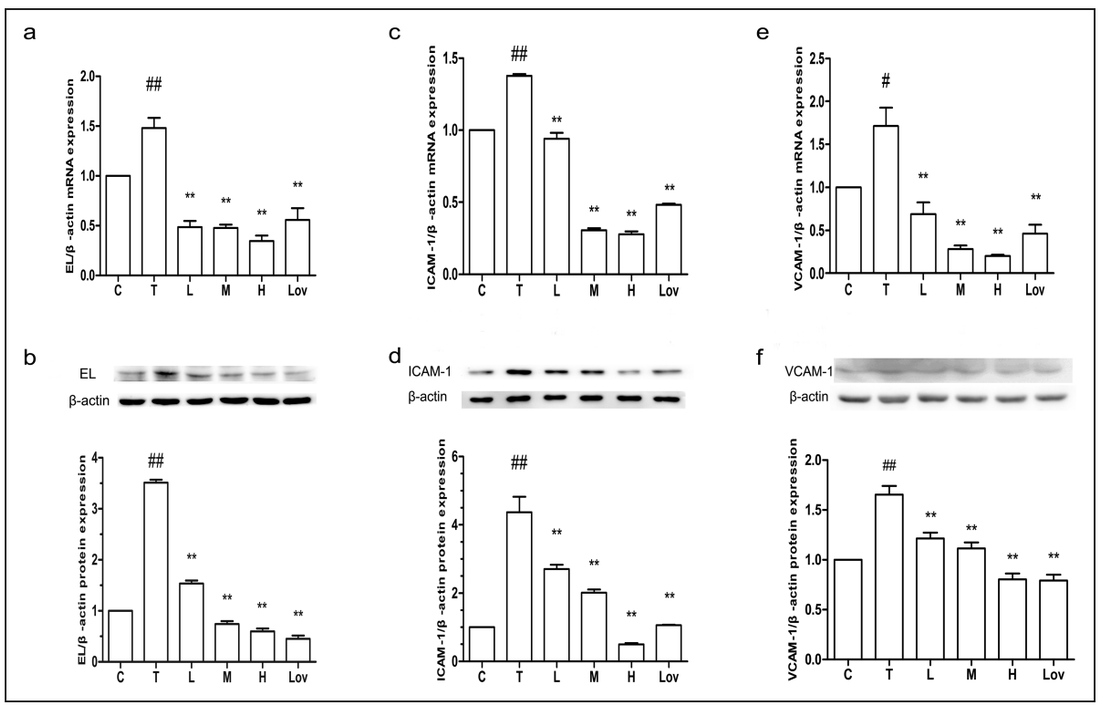

Fig. 4: Effects of naringin on EL and inflammatory cytokines expressions in HUVECs. HUVECs were incubated in medium containing TNF-α (10 ng/mL) and naringin (12.5, 25, 50 μg/mL) or lovastatin (0.05 μM) for 24 h. (a) The mRNA expression of EL in HUVECs determined by qPCR. (b) The protein expression of EL in HUVECs determined by Western blotting. (c) The mRNA expression of ICAM-1 in HUVECs determined by qPCR. (d) The protein expression of ICAM-1 in HUVECs determined by Western blotting. (e) The mRNA expression of VCAM-1 in HUVECs determined by qPCR. (f) The protein expressions of VCAM-1 in HUVECs determined by Western blotting. Each bar represents the mean ± SEM of triplicate determinations. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to HC group.

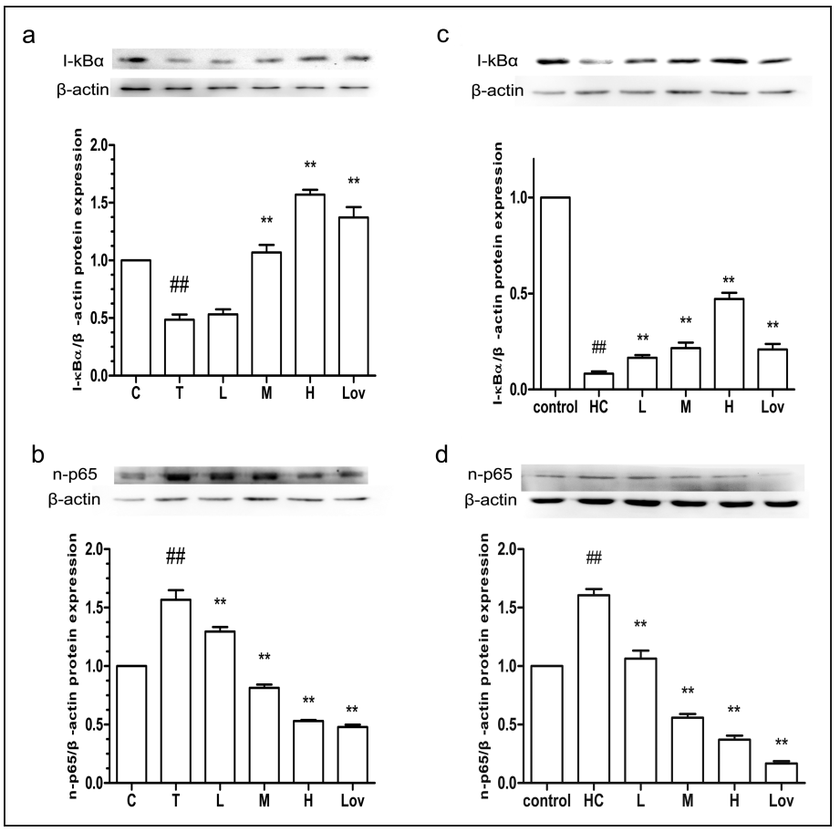

Fig. 5: Effects of naringin on NF-κB activation in HUVECs and HepG2 cells. HUVECs were incubated in medium containing TNF-α (10 ng/mL) and naringin (12.5, 25, 50 μg/mL) or lovastatin (0.05 μM) for 24 h. HepG2 cells were incubated in medium containing cholesterol (10 μg/mL) and 25-OH-cholesterol (1 μg/mL) for 24 h, and then were treated with naringin (25, 50, 100 μg/mL) or lovastatin (1 μM) for 24 h. (a) The protein expression of cytoplasmic I-κBα in HUVECs determined by Western blotting. (b) The protein expressions of nuclear p65 in HUVECs determined by Western blotting. (c) The protein expression of cytoplasmic I-κBα in HepG2 cells determined by Western blotting. (d) The protein expressions of nuclear p65 in HepG2 cells determined by Western blotting. Each bar represents the mean ± SEM of triplicate determinations. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to control; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to HC group.

3. Discussion

Naringin has been empirically proved to have little side effects, and a growing number of studies are dealing with the therapeutic use of naringin for the prevention or treatment of diseases (Chen et al. 2014; Lee et al. 2008). As an active compound existing in citrus fruits, naringin was shown to be an effective anti-cancer, anti-oxidative and anti-atherogenic agent *in vitro* and *in vivo* (Chanet et al. 2012; Lee et al. 2009; Safari et al. 2003). However, little is known concerning the cholesterol management and anti-inflammatory effects of naringin. HepG2 cells stimulated with cholesterol and 25-OH-cholesterol and HUVECs induced by TNF-α exhibited the characteristics associated with hypercholesterolemia and inflammatory stress (Mullen et al. 2004; Xu et al. 2014). Therefore in the present study we examined the mechanisms of naringin affecting cholesterol metabolism and inflammation in human hepatoma HepG2 cells and HUVECs.

Cholesterol is essential to our body but too much cholesterol termed hypercholesterolemia can lead to many diseases including arteriosclerosis. Reductions in LDL-C have been demonstrated to decrease atherosclerotic-related morbidity and mortality, and strategies aimed at lowering LDL-C remain a primary approach for atherosclerotic risk reduction (Chen et al. 2012). In most metabolic states, LDL-C is catabolized by the liver *via* hepatocyte cell surface LDLR (Melone et al. 2012). In the present study, it was found that the levels of LDLR mRNA and protein were eminently increased at HepG2 cells treated by naringin compared with the cells irritated by HC alone. LDLR expression in the liver is known to be regulated predominantly at the transcriptional level through SREBP2 and regulated by intracellular cholesterol level through a negative feedback mechanism (Moon et al. 2012). While cellular cholesterol level is low, SREBP2 is proteolytically processed to a mature and nuclear form of SREBP2 in the endoplasmic reticulum to the Golgi apparatus, and then activates LDLR transcription (Moon et al. 2012). In the present study, in response to the stimulus of cholesterol, the expression of SREBP2 was quantitatively reduced and the capability of nuclear translocation was also suppressed and under the premise of promoting the level of SREBP2 mRNA, naringin treatment for 24 h not only significantly promoted the expression of nuclear SREBP2, but also promoted the expression of cytoplasmic SREBP2 partly. Thus, we hypothesized that naringin not only improved the synthesis of SREBP2, but also promoted it's activity effectively, thereby regulating the expression of LDLR. The results showed that naringin could promote reverse cholesterol transport by enhancing the expression of LDLR in HepG2 cells to promote the elimination of cholesterol.

Then we examined whether naringin may inhibit cholesterol accumulation in HepG2 cells by regulating free cholesterol metabolism. Therefore we investigated whether the expression of CYP7A1, a rate-limiting enzyme contributing to the conversion of free cholesterol to bile acids in liver would be modulated by naringin in HepG2 cells (Defesche 2004). As show in Fig. 2a and 2b, naringin inhibited the reduction of CYP7A1 expression caused by cholesterol and 25-OH-cholesterol in HepG2 cells, and the result showed that naringin had significant beneficial

effects on the clearance of cholesterol. Naringin improved the expressions of LDLR and CYP7A1, suggesting that naringin helps to remove harmful cholesterol from the circulatory system and also contributes to cholesterol metabolism in liver.

The present study showed that naringin induced the expression of PPAR-γ which plays a major regulatory role in adipogenesis and regulates the expression of genes involved in lipid metabolism. It was demonstrated that PPAR-γ increased hepatic LDLR expression and induced the LDLR functions, such as up-regulating LDL uptake in HepG2 cells and decreasing LDL cholesterol levels in serum (Duan et al. 2012). Moreover PPAR-γ activation also induces CYP7A1 expression (Duan et al. 2012). In addition, PPAR-γ can be phosphorylated and reduced by ERK1/2 (Duan et al. 2012), and ERK1/2 phosphorylation decrease by naringin had been confirmed in this study. Thus, it is likely that naringin affects the expressions and functions of CYP7A1 and LDLR by up-regulating the expression of PPAR-γ and inhibiting ERK1/2 activation, thereby promoting the metabolism of excess cholesterol from hepatocytes.

Damage due to hyperlipidemia intensifies the susceptibility of the liver to inflammation (Jaya et al. 2010). The results showed that the over-expression of CRP induced by cholesterol and 25-OH-cholesterol could be inhibited by addition of naringin. Naringin was also effective at reducing the expression of TNF-α in a concentration-dependent manner. TNF-α CRP, are currently the best validated inflammatory biomarkers (Zhu et al. 2013). That illustrates that naringin suppressed the production of inflammatory markers in HepG2 cells caused by cholesterol and 25-OH-cholesterol. Based on that, we tested the expressions of ICAM-1 and VCAM-1 which are considered as key factors in the onset of atherosclerosis, and the results showed that naringin could obviously reduce mRNA and protein levels of ICAM-1 and VCAM-1 in HepG2 cells. Furthermore, naringin effectively restrained the NF-κB-p65 nuclear transfer and inhibited IκBα degradation in a concentration-dependent manner, illustrating that naringin inhibited NF-κB activation significantly. It was reported the gene expressions of ICAM-1 and VCAM-1 were regulated by transcriptional activation of NF-κB (Yuan et al. 2013). It was very likely that naringin's anti-inflammatory effects were partly due to NF-κB inhibition.

High levels of cholesterol can directly damage the arterial wall, leading to vascular endothelial lesion and accelerating arterial atherosclerosis (Nymo et al. 2014). Therefore the effects of naringin on TNF-α-treated HUVECs were explored as well. As a carrier of lipid which is negatively correlated with the incidence of coronary artery disease, HDL plays an important role in protecting against atherosclerosis by removing cholesterol from atheroma and transporting it back to the liver (Matsuura et al. 2007). It is generally hypothesized that variations in plasma HDL-C levels are determined by both the rate at which HDL-C is generated and the rate of catabolism of HDL particles (Ishida et al. 2003). As a member of the triacylglycerol lipase family, EL decreases HDL-C levels leading to an increased risk of atherosclerosis (Kivelä et al. 2010). The results in the current study made clear that naringin had a strong effect to inhibit EL expression in TNF-α-induced HUVECs. Therefore, we hypothesized that naringin might maintain HDL-C levels by inhibiting the expression of EL.

Inflammation has also become a crucial focus in atheroma development and endothelial cell activation is considered as necessary for the recruitment of inflammatory cells to the plaque and EL is also up-regulated in inflammatory states (Kivelä et al. 2012; Nymo et al. 2014). It was reported that EL also promoted monocyte adhesion to the vascular endothelium through an interaction with heparan sulfate proteoglycans (Paradis et al. 2006). Thus, EL may play a key role in the vicious cycle of inflammation at the site of atherosclerotic lesions (Yasuda et al. 2010). Therefore, we concluded that naringin not only regulated cholesterol metabolism but also produced indirect anti-inflammatory effects by suppressing EL expression. And then we tested expressions of ICMA-1 and VCAM-1 in HUVECs as well. The results showed that naringin obviously reduced mRNA and protein levels of ICAM-1 and VCAM-1. The gene expressions of ICAM-1 and VCAM-1 which are important factors in the activation of inflammatory cells are regulated by transcriptional activation of NF-κB (Yuan et al. 2013). Also Hans Kestler and Sybille Kempe presented that binding sites interacting with NF-κB *in vitro* and *in vivo* were identified and co-transfection experiments verified the direct regulation of the EL promoter by NF-κB (Kempe et al. 2005). The results showed that naringin effectively restrained the NF-κB-p65 nuclear transfer and inhibited IκBα degradation in HUVECs and this illustrated that naringin inhibited NF-κB activation significantly. Therefore, we summarized three following observations to explain the mechanisms underlying the protective effects of naringin against inflammation: (1) naringin could inhibit inflammation through down-regulating the expressions of ICAM-1 and VCAM-1 directly; (2) naringin could inhibit inflammation through down-regulating the expression of EL; (3) naringin could lower the expressions of EL, ICAM-1 and VCAM-1 via NF-κB pathway.

It is noteworthy that, in the present study we confirmed that lovastatin inhibited the expressions of CAMs and the activation of NF-κB in TNF-α activated HUVECs, and this is quite different from the observations and perspective which Landsberger presented in 2007 (Landsberger et al. 2007). We speculate that this is a controversial issue which should be further investigated.

The results in the present study suggested that naringin promoted reverse cholesterol transport and regulated cholesterol metabolism, and receded cholesterol accumulation-induced inflammatory response which is responsible for further cholesterol accumulation in HepG2 hepatocyte and HUVECs. In this study the major findings in support of naringin include: (1) naringin reinforced metabolism and elimination pathways for regulating cholesterol homeostasis; (2) naringin reduced over-expression of inflammatory factors in HepG2 cells and HUVECs, and contributed to the inhibition of vascular and hepatic inflammation. In summary, naringin participated in cholesterol homeostasis regulation and inhibited inflammatory signaling. These findings supported that naringin might have preventive actions against hypercholesterolemia.

4. Experimental

4.1. Cell culture

HepG2 cells (a human hepatoma cell line) and HUVECs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified, 5% CO_2/95% air atmosphere. And HepG2 cells were divided into six groups: control group, cholesterol and 25-OH-cholesterol (HC) treatment group (cholesterol 10 μg/ml and 25-OH-cholesterol 1 μg/ml), low concentration of naringin group (cholesterol 10 μg/ml and 25-OH-cholesterol 1 μg/ml + naringin 25 μg/m), medium concentration of naringin group (cholesterol 10 μg/ml and 25-OH-cholesterol 1 μg/ml + naringin 50 μg/m), high concentration of naringin group (cholesterol 10 μg/ml and 25-OH-cholesterol 1 μg/ml + naringin 100 μg/m), positive control group (lovastatin 1 μM). HUVECs were divided into six groups: control group, TNF-α treatment group (10 ng/ml), low concentration of naringin group (TNF-α 10 ng/ml + naringin 12.5 μg/m), medium concentration of naringin group (TNF-α 10 ng/ml + naringin 25 μg/m), high concentration of naringin group (TNF-α 10 ng/ml + naringin 50 μg/m), positive control group (lovastatin 0.05 μM).

4.2. Chemicals

Naringin (purity > 98% by HPLC) and lovastatin (purity > 98% by HPLC) were purchased from the National Institute for Food and Drug control.

MDA, GSH and SOD measuring kits were from Nanjing Jiancheng Bioengineering Institute. ERL1/2 (L352) polyclonal antibody and p-ERK1/2 (T202/Y204) polyclonal antibody were purchased from Bioworld Technology. CRP Monoclonal antibody and CYP7A1 polyclonal antibody were purchased from Abcam Ltd. SREBP2 polyclonal antibody, NF-κB/p65 polyclonal antibody and ICAM-1 polyclonal antibody were all from Proteintech Group Inc. TNF-α antibody was purchased from Cell Signaling Technology, Inc. EL polyclonal antibody and LDLR polyclonal antibody were purchased from Santa Cruz Biotechnology Inc. I-κBα polyclonal antibody, VCAM-1 polyclonal antibody, mouse anti-beta actin monoclonal antibody, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP and rabbit anti-goat IgG-HRP were purchased from Zhongshan Golden Bridge Biotechnology Co, Ltd.

4.3. Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis

Total RNA was isolated with RNAiso Plus (TaKaRa) from cells. 1 mg total RNA (0.8 μg/μL) was reverse transcribed with PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa) then performed by TC-512 (TECHNE). qRT-PCR analysis was performed using 7500 Real Time PCR System (Applied Biosystems) with SYBR Premix Ex TaqTM (Takara). The primers designed and synthesized by TaKaRa. Primer sequences used for q-PCR were specific to human LDLR (F 5'-GACCCAACAAGTTCAAGTGTCACAG-3', R 5'-TCATTGCAGACGTGGGAA CAG-3'); SREBP2 (F 5'- ATGCGGCTCTGGCCTATCAC-3', R 5'-CTGCACATTC AGCCAGGTTCA-3'); CYP7-A1 (F 5'- AGAGAGCTTGAGGCACGAGAA-3', R 5'- AATGGTGTTTGCTTGCGATG-3'); ICAM-1 (F 5'- TGTATGAACTGAGCAATGT GCAAGA-3', R 5'- CACCTGGCAGCGTAGGGTAA-3'); VCAM-1 (F 5'-GGCGCCTATACCATCCGAAA-3', R 5'-AGAGCACGAGAAGCTCAGGAGAA-3'); EL (F 5'-CTTTCACGAAGTTGCCTGCATA-3', R 5'-GGACACAGCATTGCC AGGA-3') and β-actin (F 5'-TGGCACCCAGCACAATGAA-3', R 5'-CTAAGTCAT AGTCCGCCTAGAGGCA-3').

4.4. Western blotting analysis

Protein was extracted from cells using the SDS lysis buffer (BeyotimeBiotech) and transferred onto a polyvinylidene difluoride (PVDF) (Immobilon) membrane after separated on SDS-polyacrylamide gelelectrophoresis (SDS-PAGE). After blocking with 5% fat-free milk the membrane was incubated with primary antibodies overnight at 4°C and secondary antibody at room temperature for 2 h. The immunostained protein was reflected by enhanced chemiluminescence (ECL) (BeyotimeBiotech), and analyzed by A BioSpectrum-410 multispectral imaging system with Chemi HR camera 410 (UVP).

4.5. Statistics

Data were expressed as mean ± standard error of mean (SEM). Results were statistically analyzed using one-way analysis of variance (ANOVA) with post hoc LSD test on Sigma Stat Advisory Statistical Software (Sigma Stat version 14.0, SPSS Inc .). Significance was defined as $p < 0.05$.

Acknowledgment: This study was supported in part by Grants from the National Natural Science Foundation of China (No. 81273508).

References

Brown JD, Plutzky J (2007) Peroxisome proliferator-activated receptors as transcriptional nodal points and therapeutic targets. Circulation 115: 518–533.

Chanet A, Milenkovic D, Deval C, Potier M, Constans J, Mazur A, Bennetau-Pelissero C, Morand C, Bérard AM (2012) Naringin, the major grapefruit flavonoid, specifically affects atherosclerosis development in diet-induced hypercholesterolemia in mice. J Nutr Biochem 23: 469–477.

Chen J, Guo R, Yan H, Tian L, You Q, Li S, Huang R, Wu K (2014) Naringin inhibits ROS-activated MAPK pathway in high glucose-induced injuries in H9c2 cardiac cells. Basic Clin Pharmacol Toxicol 114: 293–304.

Chen Q, Wang E, Ma L, Zhai P (2012) Dietary resveratrol increases the expression of hepatic 7alpha-hydroxylase and ameliorates hypercholesterolemia in high-fat fed C57BL/6J mice. Lipids Health Dis 11: 56.

Davidson MH, Palmisano J, Wilson H, Liss C, Dicklin MR (2003) A multicenter, randomized, double-blind clinical trial comparing the low-density lipoprotein cholesterol-lowering ability of lovastatin 10, 20, and 40 mg/d with fluvastatin 20 and 40 mg/d. Clin Ther 25: 2738–2753.

Defesche JC (2004) Low-density lipoprotein receptor–its structure, function, and mutations. Semin Vasc Med 4: 5–11.

Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J (2009) Major lipids, apolipoproteins, and risk of vascular disease. JAMA 302: 1993–2000.

Duan Y, Chen Y, Hu W, Li X, Yang X, Zhou X, Yin Z, Kong D, Yao Z, Hajjar DP, Liu L, Liu Q, Han J (2012) Peroxisome proliferator-activated receptor gamma activation by ligands and dephosphorylation induces proprotein convertase subtilisin kexin type 9 and low density lipoprotein receptor expression. J Biol Chem 287: 23667–23677.

E L, Fragakis N, Ioannidou E, Bounda A, Theodoridou S, Klonizakis P, Garipidou V (2010) Increased levels of proinflammatory cytokines in children with family history of coronary artery disease. Clinical Cardiol 33: E6–10.

Hirata K, Dichek HL, Cioffi JA, Choi SY, Leeper NJ, Quintana L, Kronmal GS, Cooper AD, Quertermous T (1999) Cloning of a unique lipase from endothelial cells extends the lipase gene family. J Biol Chem 274: 14170–14175.

Horton JD, Goldstein JL, Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109: 1125–1131.

Ishida T, Choi S, Kundu RK, Hirata K, Rubin EM, Cooper AD, Quertermous T (2003) Endothelial lipase is a major determinant of HDL level. J Clin Invest 111: 347–355.

Jain M, Parmar HS (2011) Evaluation of antioxidative and anti-inflammatory potential of hesperidin and naringin on the rat air pouch model of inflammation. Inflamm Res 60: 483–491.

Jaya C, Venkatesan V, Anuradha CV (2010) Inflammatory responses in liver induced by high fat plus fructose diet: therapeutic potential of cissus quadrangularis stem. Int J Biol Med Res 1: 120–124.

Jaye M, Lynch KJ, Krawiec J, Marchadier D, Maugeais C, Doan K, South V, Amin D, Perrone M, Rader DJ (1999) A novel endothelial-derived lipase that modulates HDL metabolism. Nat Genet 21: 424–428.

Jeon SM, Park YB, Choi MS (2004) Antihypercholesterolemic property of naringin alters plasma and tissue lipids, cholesterol-regulating enzymes, fecal sterol and tissue morphology in rabbits. Clin Nutr 23: 1025–1034.

Kempe S, Kestler H, Lasar A, Wirth T (2005) NF-kappaB controls the global pro-inflammatory response in endothelial cells: evidence for the regulation of a pro-atherogenic program. Nucleic Acids Res 33: 5308–5319.

Kim S, Kim CK, Lee KS, Kim JH, Hwang H, Jeoung D, Choe J, Won MH, Lee H, Ha KS, Kwon YG, Kim YM (2013) Aqueous extract of unripe Rubus coreanus fruit attenuates atherosclerosis by improving blood lipid profile and inhibiting NF-kappaB activation via phase II gene expression. J Ethnopharmacol 146: 515–524.

Kivelä AM, Mäkinen PI, Jyrkkänen HK, Mella-Aho E, Xia Y, Kansanen E, Leinonen H, Verma IM, Ylä-Herttuala S, Levonen A (2010) Sulforaphane inhibits endothelial lipase expression through NF-kappaB in endothelial cells. Atherosclerosis 213: 122–128.

Kivelä AM, Dijkstra MH, Heinonen SE, Gurzeler E, Jauhiainen S, Levonen AL, Yla-Herttuala S (2012) Regulation of endothelial lipase and systemic HDL cholesterol levels by SREBPs and VEGF-A. Atherosclerosis 225: 335–340.

Kwak DH, Lee JH, Kim T, Ahn HS, Cho WK, Ha H, Hwang YH, Ma JY (2012) Aristolochia manshuriensis Kom inhibits adipocyte differentiation by regulation of ERK1/2 and Akt pathway. PLoS One 7: e49530.

Landsberger M, Wolff B, Jantzen F, Rosenstengel C, Vogelgesang D, Staudt A, Dahm JB, Felix SB (2007) Cerivastatin reduces cytokine-induced surface expression of ICAM-1 via increased shedding in human endothelial cells. Atherosclerosis 190: 43–52.

Lee EJ, Kim DI, Kim WJ, Moon SK (2009) Naringin inhibits matrix metalloproteinase-9 expression and AKT phosphorylation in tumor necrosis factor-alpha-induced vascular smooth muscle cells. Mol Nutr Food Res 53: 1582–1591.

Lee EJ, Moon GS, Choi WS, Kim WJ, Moon SK (2008) Naringin-induced p21WAF1-mediated G(1)-phase cell cycle arrest via activation of the Ras/Raf/ERK signaling pathway in vascular smooth muscle cells. Food Chem Toxicol 46: 3800–3807.

Li H, Ooi SQ, Heng CK (2013) The role of NF-small ka, CyrillicB in SAA-induced peroxisome proliferator-activated receptor gamma activation. Atherosclerosis 227: 72–78.

Libby P, Ridker PM, Maseri A (2002) Inflammation and atherosclerosis. Circulation 105: 1135–1143.

Lusis AJ (2000) Atherosclerosis. Nature 407: 233–241.

Matsuura F, Oku H, Koseki M, Sandoval JC, Yuasa-Kawase M, Tsubakio-Yamamoto K, Masuda D, Maeda N, Tsujii K, Ishigami M, Nishida M, Hirano K, Kihara S, Hori M, Shimomura I, Yamashita S (2007) Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. Biochem Biophys Res Comm 358: 1091–1095.

Maugeais C, Tietge UJ, Broedl UC, Marchadier D, Cain W, McCoy MG, Lund-Katz S, Glick JM, Rader DJ (2003) Dose-dependent acceleration of high-density lipoprotein catabolism by endothelial lipase. Circulation 108: 2121–2126.

Melone M, Wilsie L, Palyha O, Strack A, Rashid S (2012) Discovery of a new role of human resistin in hepatocyte low-density lipoprotein receptor suppression mediated in part by proprotein convertase subtilisin/kexin type 9. J Am Coll Cardiol 59: 1697–1705.

Moon J, Lee SM, Do HJ, Cho Y, Chung JH, Shin MJ (2012) Quercetin up-regulates LDL receptor expression in HepG2 cells Phytother Res 26: 1688–1694.

Mullen E, Brown RM, Osborne TF, Shay NF (2004) Soy isoflavones affect sterol regulatory element binding proteins (SREBPs) and SREBP-regulated genes in HepG2 cells. J Nutr 134: 2942–2947.

Norata GD (2014) [PCSK9 inhibitors and dyslipidemias: an update on clinical evidence] G Ital Cardiol (Rome) 15: 301–305.

Nymo S, Niyonzima N, Espevik T, Mollnes TE (2014) Cholesterol crystal-induced endothelial cell activation is complement-dependent and mediated by TNF. Immunobiology 219: 786–792.

Paradis ME, Badellino KO, Rader DJ, Deshaies Y, Couture P, Archer WR, Bergeron N, Lamarche B (2006) Endothelial lipase is associated with inflammation in humans. J Lipid Res 47: 2808–2813.

Ren S, Ning Y (2014) Sulfation of 25-hydroxycholesterol regulates lipid metabolism, inflammatory responses, and cell proliferation. Am J Physiol Endocrinol Metab 306: E123–130.

Safari MR, Rezaie M, Sheikh N (2003) Effects of some flavonoids on the susceptibility of low-density lipoprotein to oxidative modification. Indian J Biochem Biophys 40: 358–361.

Sasaki CY, Barberi TJ, Ghosh P, Longo DL (2005) Phosphorylation of RelA/p65 on serine 536 defines an I{kappa}B{alpha}-independent NF-{kappa}B pathway. J Biol Chem 280: 34538–34547.

Vega GL, Grundy SM (1996) Hypoalphalipoproteinemia (low high density lipoprotein) as a risk factor for coronary heart disease. Curr Opin Lipidol 7: 209–216.

Weber LW, Boll M, Stampfl A (2004) Maintaining cholesterol homeostasis: sterol regulatory element-binding proteins. World J Gastroenterol 10: 3081–3087.

Wu S, Xu H, Peng J, Wang C, Jin Y, Liu K, Sun H, Qin J (2015) Potent anti-inflammatory effect of dioscin mediated by suppression of TNF-alpha-induced VCAM-1, ICAM-1and EL expression via the NF-kappaB pathway. Biochimie 110: 62–72.

Wu X, Huang H, Tang F, Le K, Xu S, Liu P (2010) Regulated expression of endothelial lipase in atherosclerosis. Mol Cell Endocrinol 315: 233–238.

Xu Q, Luan T, Fu S, Yang J, Jiang C, Xia F (2014) Effects of pitavastatin on the expression of VCAM-1 and its target gene miR-126 in cultured human umbilical vein endothelial cells. Cardiovasc Ther 32: 193–197.

Yasuda T, Hirata K, Ishida T, Kojima Y, Tanaka H, Okada T, Quertermous T, Yokoyama M (2007) Endothelial lipase is increased by inflammation and promotes LDL uptake in macrophages. J Atheroscler Thromb 14: 192–201.

Yasuda T, Ishida T, Rader DJ (2010) Update on the role of endothelial lipase in high-density lipoprotein metabolism, reverse cholesterol transport, and atherosclerosis. Circulation J 74: 2263–2270.

Yuan Q, Zhang X, Liu Z, Song S, Xue P, Wang J, Ruan J (2013) Ethanol extract of Adiantum capillus-veneris L. suppresses the production of inflammatory mediators by inhibiting NF-kappaB activation. J Ethnopharmacol 147: 603–611.

Zhu Y, Ling W, Guo H, Song F, Ye Q, Zou T, Li D, Zhang Y, Li G, Xiao Y, Liu F, Li Z, Shi Z, Yang Y (2013) Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: a randomized controlled trial. Nutr Metabol Cardiovasc Dis 23: 843–849.