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Simvastatin inhibits ox-LDL-induced inflammatory adipokines secretion via amelioration of ER stress in 3T3-L1 adipocyte

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ABSTRACT

Adipocytes behave as a rich source of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1). Endoplasmic reticulum (ER) stress in adipocytes can alter adipokines secretion and induce inflammation. The aim of this study is to evaluate the effect of simvastatin on the ox-LDL-induced ER stress and expression and secretion of TNF-α and MCP-1 in 3T3-L1 adipocytes. Differentiated adipocytes were treated with various concentrations of ox-LDL (0–100 μg/ml) for 24 h with or without simvastatin pre-treatment. The protein expressions of ER stress markers, glucose-regulated protein 78 (GRP78) and C/EBP homology protein (CHOP), were determined by Western blot analysis. The mRNA expressions of TNF-α and MCP-1 were measured by real-time PCR. The protein release of TNF-α and MCP-1 in culture medium were evaluated by ELISA. Ox-LDL treatment led to significant up-regulation of GRP78 and CHOP in dose-dependent manner. The expressions of TNF-α and MCP-1 were dose-dependently increased at mRNA and protein levels after ox-LDL intervention. The effects of ox-LDL on adipocytes were abolished by pre-treatment with 4-phenylbutyrate (4-PBA), a chemical chaperone known to ameliorate ER stress. Simvastatin could inhibit ox-LDL-induced ER stress and reduce the expression of TNF-α and MCP-1 at mRNA and protien level in dose dependent manner. In conclusion, ox-LDL can stimulate the expression and secretion of TNF-α and MCP-1 through its activation of ER stress in adipocytes. Simvastatin might exert direct anti-inflammatory effects in adipocytes through amelioration of ER stress.

1. Introduction

In addition to store excess energy in form of triglyceride, adipose tissue is an active endocrine organ and serves as a rich source of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein 1 (MCP-1) and interleukin-6 (IL-6) [1]. Adipocytes have substantial ability to synthesize and secrete TNF-α and MCP-1 [2,3]. The adipose expression and secretion of both adipokines are elevated in obese state, which may at least partly account for the chronic low-grade inflammation in obesity. It has been demonstrated that inflammation plays a pivotal role in the pathogenesis of insulin resistance and atherosclerosis associated with obesity [4]. The abnormalities of the expression and secretion of adipokines may be the link between obesity and its complications, which will be a potential target for the treatment of obesity.

Endoplasmic reticulum (ER) is a central organelle for proteins synthesis, folding and maturation. Various genetic and environmental insults may lead to accumulation of unfolded or misfolded proteins in the ER lumen, causing ER stress. Prolonged ER stress may impair the metabolism and functions of cells. The ER of adipocytes plays a major role in the assembly and secretion of adipokines. Recent studies have reported that ER stress is increased in adipose tissue of obese mice and human subjects [5,6]. It has been confirmed that ER stress in adipocytes can modify adipokines secretion and induce inflammation [7,8]. So it could be presumed that inhibition of ER stress may be an effective approach to reduce the risk of obesity and its complications.

Oxidized low density lipoprotein (ox-LDL) is considered a major player in the pathogenesis of atherosclerosis [9]. Circulating ox-LDL is significantly correlated with most of the cardiovascular risk factors including dyslipidemia, type-2 diabetes, obesity, and metabolic syndrome [10]. Ox-LDL has a wide range of atherogenic properties including up-regulation of inflammatory genes, increased expression of adhesion molecules on endothelial cells, monocyte chemotaxis and destabilization of plaques. Recent studies indicated that ox-LDL could trigger ER stress in endothelial cells and macrophages [11,12]. However it is rarely reported about the effect of ox-LDL on ER stress and subsequent adipokines secretion in adipocytes.

Statins, potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, significantly reduce serum

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cholesterol level and decrease the incidence of coronary heart disease [13]. The overall clinical benefits of statins appear to be beyond the cholesterol-lowering effects, suggesting that statins have pleiotropic effects including anti-inflammatory property [14]. Recently it has been found that statins significantly inhibit ER stress in cardiomyocytes and macrophages [15–17], whereas it is still unknown about the effect of statins on ER stress of adipocytes. So the aim of this study is to determine the effect of simvastatin on adipocytes ER stress and secretion of inflammatory adipokines, TNF-α and MCP-1.

2. Materials and methods

2.1. Cell culture and treatment

3T3-L1 preadipocytes were cultured and induced to differentiate into mature adipocytes as described previously [18]. Differentiated adipocytes were serum starved for 18 h in DMEM supplemented with 0.2% bovine serum albumin (BSA) before treatment. For the experiment, adipocytes were exposed to various concentration of ox-LDL (0–100 μg/ml) for 24 h. To further verify whether the effect of ox-LDL on adipocytes is associated with ER stress activation, the adipocytes were pretreated for 12 h with various doses of 4-phenylbutyrate (4-PBA) (0–20 mM), a chemical chaperone known to ameliorate ER stress [19], and then stimulated with 50 μg/ml of ox-LDL for 24 h. For simvastatin studies, 3T3-L1 adipocytes were treated with various concentrations of simvastatin (0–10 μmol/L) for 12 h, followed by treatment with 50 μg/ml of ox-LDL for 24 h. At the end of the study, the supernatants and monolayer cells were harvested for next experiments.

2.2. Western blot analysis

ER stress is characterized by the expression of ER stress indicators, glucose-regulated protein 78 (GRP78) and C/EBP homology protein (CHOP). GRP78 is a chaperone in ER and plays a crucial role in the regulation of the ER dynamic equilibrium. CHOP is a major transcriptional factor responsible for ER stress-induced apoptosis. Both GRP78 and CHOP are significantly up-regulated when ER stress occurs. The protein expression of CHOP and GRP78 were determined by Western blot analysis. Briefly, cultured cells were lysed in radio immunoprecipitation assay buffer (RIPA, Beyotime Institute of Biotechnology, China). Equivalent amounts of protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes. The membrane was incubated with specific monoclonal anti-GRP78 or anti-CHOP primary antibodies (Santa Cruz Biotechnology, USA) at 4 °C for overnight. After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma), immunoreactive bands were visualized using the enhanced chemiluminescence detection system. Data were quantified by densitometry after scanning using the TINA software (Raytest, Germany). The expression of GRP78 and CHOP was evaluated and compared with the expression of β-actin.

2.3. RNA isolation and real-time PCR

The mRNA expressions of TNF-α and MCP-1 were evaluated by the method of real-time PCR. Total RNA was extracted from adipocytes using Trizol reagent (Invitrogen) according to manufacturer's instructions. RNA was reverse transcribed using SuperScript III First-Strand Synthesis Supermix (Invitrogen). The cDNA samples were amplified in duplicate in 96-microtiter plates (Applied Biosystems). Each PCR reaction (20 μl of total volume) contained: 10 μl of SYBR Green PCR Master Mix (Applied Biosystems), 5 pmols of each primer, 1 μg of cDNA. The PCR primers were the following: (1) TNF-α: 5′-TTC TAT GGC CCA GAC CCT CA-3′ and 5′-ACT TGG TGG TTT GCT ACG ACG-3′; (2) MCP-1: 5′-GCA GGT CCC TGT CAT GCT TC-3′ and 5′-GAG TGG GGC GTT AAC TGC AT-3′. Real-time PCR reactions were carried out in an ABI PRISM 7500 real-time PCR apparatus. The thermal profile settings were 95 °C for 2 min, then 40 cycles at 95 °C for 10 s, 60 °C for 30 s and 70 °C for 45 s. The relative mRNA expression levels were normalized to expression of 28S rRNA.

2.4. TNF-α and MCP-1 protein measurement

TNF-α and MCP-1 concentrations were measured in culture medium using enzyme linked immunoabsorbent assay (ELISA, R & D Systems) with a sensitivity of 1 pg/ml and no cross-reactivity against other cytokines according to the manufacturer's recommendations. Each sample was assayed in triplicate. Intra-assay and inter-assay precision variability was <8%.

2.5. Statistical analysis

Results are represented as the means ± SD. Comparisons among groups were performed by one-way ANOVA analysis. Differences were considered significant at a value of $P < 0.05$ for all tests.

3. Results

3.1. Effect of ox-LDL on ER stress and expression and secretion of TNF-α and MCP-1

In the present study, we examined the ER stress response of adipocytes after treatment with ox-LDL by measuring the protein levels of ER stress markers, GRP78 and CHOP. Incubation of adipocytes with ox-LDL led to significant up-regulation of GRP78 and CHOP in dose-dependent manner, suggesting ox-LDL may induce ER stress in adipocytes (Fig. 1). Since ER stress may alter adipokines secretion and induce inflammation, we further evaluated the effect of ox-LDL on the expression and secretion of inflammatory adipokines. It was found that the expressions of both TNF-α and MCP-1 were significantly increased at mRNA and protein levels after ox-LDL intervention (Fig. 1).

3.2. Chemical chaperone 4-PBA attenuated the effect of ox-LDL on adipocytes

4-PBA is a well-established chemical chaperone and ER stress inhibitor [19]. To further explore whether the stimulating effect of ox-LDL on adipokines secretion is attributed to the activation of ER stress, adipocytes were treated with 4-PBA before exposure to ox-LDL. As compared with adipocytes treated with ox-LDL alone, pre-treatment with 4-PBA significantly decreased the protein expression of GRP78 and CHOP in dose-dependent manner. At the same time, the mRNA expression and secretion of TNF-α and MCP-1 were also dose-dependently reduced in 4-PBA pre-treated adipocytes (Fig. 2).

3.3. Simvastatin inhibited ox-LDL-induced ER stress and inflammatory adipokines secretion in 3T3-L1 adipocytes

The effect of simvastatin on ox-LDL-induced ER stress and adipokines secretion was determined in 3T3-L1 adipocytes. As compared with adipocytes without simvastatin pre-incubation, the ox-LDL-induced expressions of GRP78 and CHOP were significantly suppressed by simvastatin pre-treatment in dose-dependent manner. The ox-LDL-induced mRNA expression and secretion of TNF-α

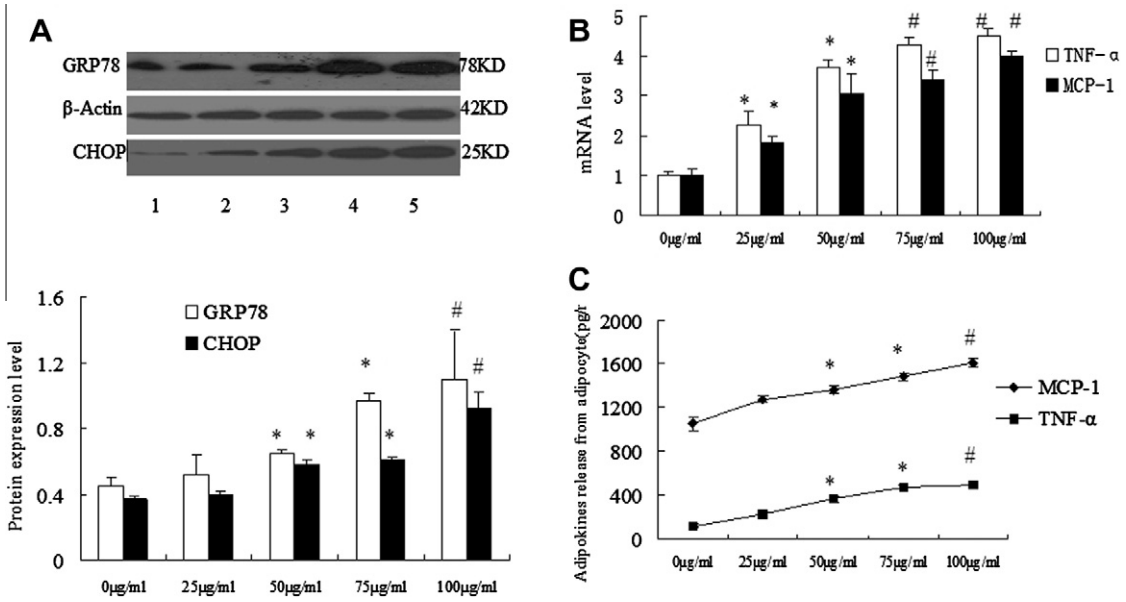

Fig. 1. Effect of ox-LDL on ER stress and expression and secretion of TNF-α and MCP-1 in 3T3-L1 adipocyte. Differentiated adipocytes were exposed to increasing concentrations of ox-LDL (0–100 μg/ml) for 24 h. (A) The protein expression of CHOP and GRP78 were determined by Western blot analysis. A representative electrophoregram and the quantification of the normalized protein levels are shown. Lane 1: control; lane 2: ox-LDL at 25 μg/ml; lane 3: ox-LDL at 50 μg/ml; lane 4: ox-LDL at 75 μg/ml; lane 5: ox-LDL at 100 μg/ml. (B) The mRNA expressions of TNF-α and MCP-1 were evaluated by real-time PCR. (C) TNF-α and MCP-1 concentrations were measured in culture medium using the method of ELISA. Values are means ± SD (n = 5). $^{*}P < 0.05$, as compared with control; $^{\#}P < 0.01$, as compared with control.

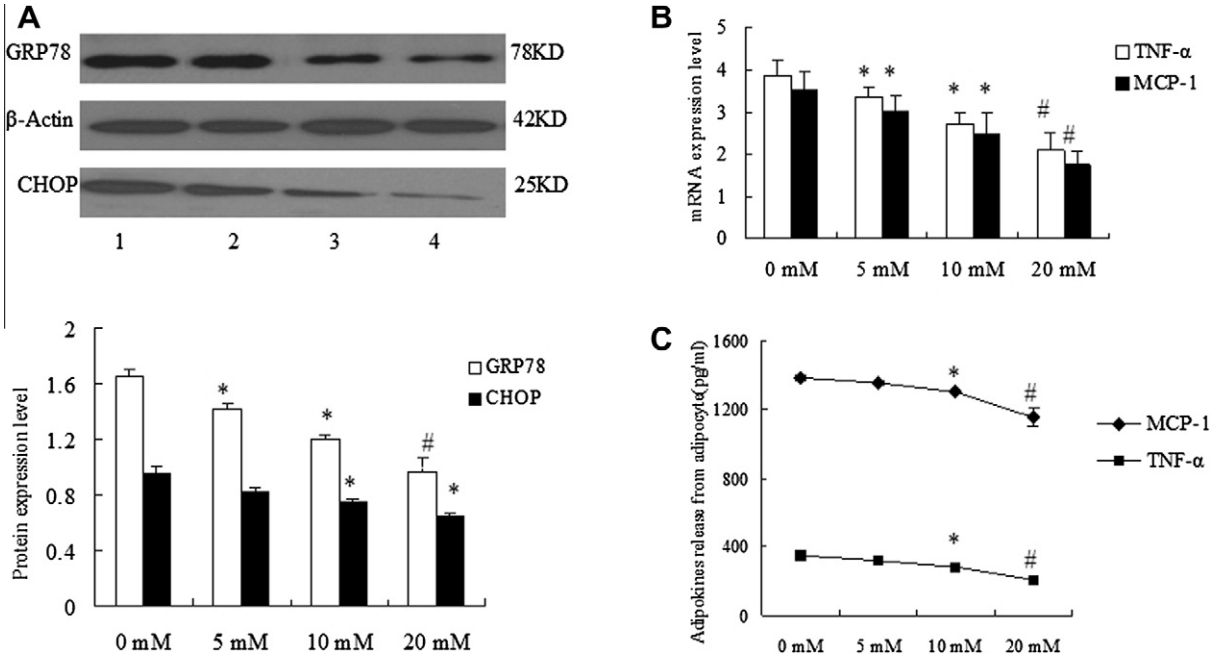

Fig. 2. Chemical chaperone 4-PBA attenuated the stimulating effect of ox-LDL on adipocytes ER stress and adipokines secretion. Differentiated adipocytes were pretreated for 12 h with various doses of 4-PBA (0–20 mM) and then stimulated with 50 μg/ml of ox-LDL for 24 h. (A) The protein expression of CHOP and GRP78 were determined by Western blot analysis. A representative electrophoregram and the quantification of the normalized protein levels are shown. Lane 1: 4-PBA 0 mM + ox-LDL 50 μg/ml; lane 2: 4-PBA 5 mM + ox-LDL 50 μg/ml; lane 3: 4-PBA 10 mM + ox-LDL 50 μg/ml; lane 4: 4-PBA 20 mM + ox-LDL 50 μg/ml. (B) The mRNA expressions of TNF-α and MCP-1 were evaluated by real-time PCR. (C) TNF-α and MCP-1 concentrations were measured in culture medium using the method of ELISA. Values are means ± SD (n = 5). $^{*}P < 0.05$, as compared with control; $^{\#}P < 0.01$, as compared with control.

and MCP-1 were also markedly inhibited by simvastatin in dose-dependent manner (Fig. 3).

4. Discussion

Ox-LDL has been demonstrated to contribute to the pathogenesis of atherosclerosis and insulin resistance. Circulating ox-LDL is significantly increased in patients with type-2 diabetes, obesity or metabolic syndrome [20,21]. Previous reports have shown the significant association between ox-LDL and circulating levels of inflammatory cytokines [22,23], the underlying mechanism for which has not been fully clarified. Adipocytes behave as inflammatory source and contribute to low grade inflammation in obesity, therefore it is meaningful to explore the direct effect of ox-LDL

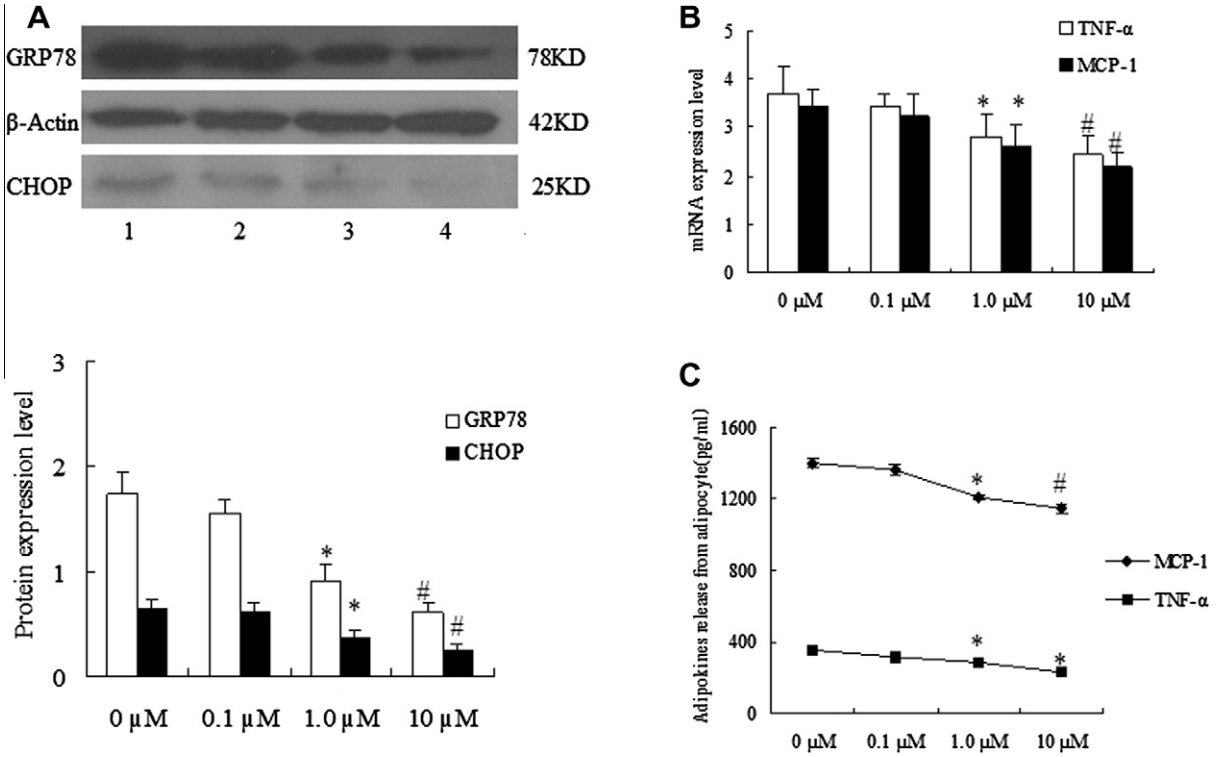

Fig. 3. Simvastatin inhibited ox-LDL-induced ER stress and inflammatory adipokines secretion in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with increasing concentrations of simvastatin (0–10 μmol/L) for 12 h, followed by treatment with 50 μg/ml of ox-LDL for 24 h. (A) The protein expression of CHOP and GRP78 were determined by Western blot analysis. A representative electrophoregram and the quantification of the normalized protein levels are shown. Lane 1: simvastatin 0 μmol/L + ox-LDL 50 μg/ml; lane 2: simvastatin 0.1 μmol/L + ox-LDL 50 μg/ml; lane 3: simvastatin 1.0 μmol/L + ox-LDL50 μg/ml; lane 4: simvastatin 10 μmol/L + ox-LDL 50 μg/ml. (B) The mRNA expressions of TNF-α and MCP-1 were evaluated by real-time PCR. (C) TNF-α and MCP-1 concentrations were measured in culture medium using the method of ELISA. Values are means ± SD (n = 5). $^{*}P < 0.05$, as compared with control; $^{\#}P < 0.01$, as compared with control.

on inflammatory adipokines secretion. Kuniyasu et al. reported that ox-LDL could increase the mRNA expression and protein secretion of plasminogen activator inhibitor-1 (PAI-1) from adipocytes [24]. Consistently, we found that ox-LDL dose-dependently stimulated the expression of TNF-α and MCP-1 at mRNA and protein level in the present study, suggesting the direct pro-inflammatory effect of ox-LDL on adipocytes. To some extent, these results may help explain the close link between ox-LDL and systemic inflammation.

ER stress observed in obesity has recently received a great deal of attention. Recent studies indicate that activation of ER stress plays a central role in regulating adipocyte function, leading to disturbed adipokines secretion and lipid metabolism in obesity [25]. Ox-LDL has been shown to trigger ER stress in endothelial cells and macrophages [11,12]. The effect of ox-LDL on ER stress of adipocytes was determined in the present study. We reported for the first time that ox-LDL significantly stimulated the adipocytes expression of GRP78 and CHOP in dose-dependent manner, suggesting that ox-LDL may also activate ER stress in adipocytes. Further study found that chemical chaperone 4-PBA could ameliorate ox-LDL-induced ER stress and subsequently attenuate the stimulating effect of ox-LDL on the secretion of TNF-α and MCP-1. The results mentioned above suggested that the ox-LDL-induced stimulation of TNF-α and MCP-1 expression and secretion might be, at least partly, due to its activation of ER stress, which supported the concept that ER stress may be the proximal cause of inflammation in adipocytes [26].

The mechanism by which ox-LDL triggered ER stress in adipocytes is complex and need to be further elucidated. Ox-LDL is a marker of lipoprotein-associated oxidative stress. Oxidative stress is a common insult that can lead to ER stress. So ox-LDL may induce ER stress through its oxidative stress properties. Previous study of Kuniyasu et al. indicated that adipocytes may function as phagocytes like macrophages to uptake and degrade ox-LDL [27]. It has been demonstrated that cholesterol overload in macrophage led to increased ER stress and inflammatory cytokines secretion, which is dependent on cholesterol trafficking to the endoplasmic reticulum [28]. Therefore it could be presumed that cholesterol load may be increased in adipocytes through endocytosis and degradation of ox-LDL, which subsequently result in the activation of ER stress. Though need further study, this provides another mechanism for the induction of adipocytes ER stress by ox-LDL in addition to oxidative stress.

We previously observed that atorvastatin reduced the expression and secretion of TNF-α and leptin in primary adipocytes isolated from hypercholesterolemia rabbits [29,30]. This suggested that statins have direct anti-inflammatory effects on adipocyte, the mechanism for which has not been fully elucidated. Though there exists conflicting report [31], statins have been shown to inhibit ER stress in several cell types such as cardiomyocytes and macrophages [15–17]. However it is still unclear whether statins have inhibitory effect on ER stress of adipocytes. We explored the impact of simvastatin on ox-LDL-induced ER stress and proinflammatory adipokines release in 3T3-L1 adipocytes in this study. We found for the first time that similar to chemical chaperone 4-PBA, simvastatin pre-treatment significantly suppressed the ox-LDL-induced expressions of GPR78 and CHOP. The ox-LDL-induced mRNA expression and secretion of TNF-α and MCP-1 were also markedly inhibited by simvastatin pre-treatment. The results suggest that simvastatin exerts anti-inflammatory actions in adipocytes at least partly through inhibition of ER stress. Amelioration of ER stress in adipocytes may be another mechanism of the pleiotropic actions of statins. These findings enrich our understanding of the beneficial effects of statins. The precise mechanism for the

effect of simvastatin on ER stress of adipocytes is not clear now. Zhao et al. reported that the inhibitory effect of pravastatin on ER stress of cardiomyocytes could be abolished by farnesyl pyrophosphate ammonium salt (FPP) or geranylgeranyl pyrophosphate ammonium salt (GGPP), metabolic products of cholesterol [15], indicating that pravastatin attenuated ER stress by inhibiting HMG-CoA reductase. It is reasonable to assume that the effect of simvastatin to inhibit ER stress of adipocytes might also be due to its inhibition of HMG-CoA reductase, which needs to be clarified in future studies.

In conclusion, the present study indicated that ox-LDL stimulated the expression and secretion of TNF-α and MCP-1 in 3T3-L1 adipocytes through activation of ER stress. Simvastatin could attenuate the stimulating effect of ox-LDL on inflammatory adipokines release via amelioration of ER stress.

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