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Simvastatin Inhibits the Proliferation and Apoptosis of Macrophages Induced by Mechanical Stress and/or Oxidized Low-Density Lipoprotein

Kefeng Liu[1,2, §], Zhengyu Zhang[1,3, §], Ting Pei[1], Ziqing Li[4], Jingjing Wang[1], Hong Wang[1], Suning Ping[1], Lie Deng[1], Linli Wang[1], Jintao Huang[5], Puyi Sheng[4], Shuying Liu[1], Chaohong Li[1]

Abbreviations: NC, negative control; MS, mechanical stress; ox-LDL (OL), oxidized low-density lipoproteins; nLDL (nL), normal low-density lipoprotein; SIM, simvastatin; Eth, ethanol; ROS, reactive oxygen species; AS, atherosclerosis; VSMCs, vascular smooth muscle cells; MAPKs, mitogen-activated protein kinases; ERK, extracellular regulated protein kinases; JNK, c-Jun N-terminal kinase; P38, *p38MAPK* kinase; 5-mc, 5-methylcytosine; *DAPI*, 4′,6-diamidino-2-phenylindole, a fluorescent stain; TUNEL, TdT-mediated dUTP nick end labeling; and Ki67, an antigen, the prototypic cell cycle related nuclear protein.

Abstract: This study was designed to investigate the effects of mechanical stress (MS) and/or oxidized low-density lipoprotein (oxLDL) on proliferation and apoptosis of RAW264.7 macrophages and the underlying mechanisms. The cultured quiescent RAW264.7 macrophages were subject to stimulation with MS and/or oxLDL in the presence or absence of simvastatin and then harvested for Western blot, and immunoflourecence. Either MS or oxLDL alone could cause increase in cell proliferation and apoptosis, while their combination led to an additive effect. In terms of mechanisms, MS and/or oxLDL significantly increased phosphorylation levels of MAPKs (ERKs, JNKs and p38MAPK), promoted the reactive oxygen species (ROS) and up-regulated DNA methylation in RAW264.7 macrophages. The increased DNA methylation was associated with

[1]Department of Histology and Embryology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510089, Guangdong, China.
[2]Department of Histology and Embryology, Xiangnan University, Chenzhou, 423000, Hunan Province, China.
[3]Guangzhou Institute of Cardiovascular Disease, the Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510260, Guangdong, China
[4]Department of orthopedic Surgery, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, 510089, Guangdong, China.
[5]Medical Experimental Center, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510089, Guangdong, China.
Correspondence should be addressed to Shuying Liu and Chaohong Li, Department of Histology and Embryology, Zhongshan School of Medicine, Sun Yat-sen University, 74# Zhongshan Road 2, Guangzhou, 510089, Guangdong Province, China. Tel.: +86-(0)20-87332488; Fax: +86-(0)20-87331451; E-mail: liushuy3@mail.sysu.edu.cn and lichaoh@mail.sysu.edu.cn
§These authors are equal contribution

proliferation but not apoptosis. In contrast, simvastatin could remarkably inhibit all the effects mentioned above. MS and oxLDL can simultaneously promote both proliferation and apoptosis of macrophages through activating MAPKs, ROS, and DNA methylation signaling, which can be directly inhibited by the simvastatin treatment. The study results can provide novel information for the pathogenesis and prevention of hypertensive mechanical stress-related vascular diseases.

Short title: Proliferation and Apoptosis of Macrophages

Keywords: Oxidative stress, apoptosis, macrophages

1 Introduction

Hypertension is a serious threat to human health. Although people's quality of life can be improved by controlling blood pressure, the rates of morbidity and mortality still shows no significant decline, especially when the condition is complicated with hyperlipidemia. Many factors may cause hypertension [Li and Xu (2007)], but once hypertension occurs, the biological mechanical stress caused by increased blood pressure becomes the main factor for vascular remodeling [Gibbons and Dzau (1994); Li, Wernig and Leitges et al. (2003)] In the early stage of hypertension, the micro-resistance arteries appear vascular sclerosis, but in the middle and late stage, large and medium arteries appear arteriosclerosis or atherosclerosis. Hyperlipidemia, hypertension and hyperglycemia are important risk factors for atherosclerosis, however, the vascular lesions occur only in the arteries rather than veins. But once the vein grafts are transplanted into the arteries, the changed structures and functions *i.e.*, vascular remodeling immediately take place and are especially more obvious in the combination of hyperglycemia or hyperlipidemia [Dietrich *et al.* (2000); Ping *et al.* (2017)]. This indicates that arterial pressure-induced signaling plays key roles in the process of vascular remodeling, which can be additively activated by hyperglycemia or hyperlipidemia. Therefore, investigation concerning the influences of blood lipids or blood sugar on the vascular wall cells must be combined with the effects of mechanical stress on the vascular wall cells. Blood flows through the vessel wall, mainly generating two types of forces, *i.e.*, shear stress and mechanical stress.[Prado *et al.* (2006)] The former produces a force parallel to the long axis of the vessel wall, playing an important role in regulating the structure and function of the tunica interna endothelial cells, while the latter produces a force perpendicular to the vessel wall, simultaneously affecting the tunica interna endothelial and tunica media smooth muscles, as well as the tunica externa fibroblasts. Reported data indicated that vascular smooth muscle cells (VSMCs), macrophages and a small amount of inflammatory cells existed in the atherosclerotic plaques at same time.[Boyle (2005); Tuttolomondo *et al.* (2012)] Thus, it is obvious that mechanical stress also plays an important role in regulating macrophages during the onset of vascular lesions. At present, studies on the effects of shear stress on the vascular cells mainly focus on the effects of endothelium, while those on mechanical stress mainly focus on VSMCs, and the influences of the mechanical stress on the macrophages in the hypertension atheromatous plaques are rarely reported.

Previous studies have shown that mechanical stress can induce non-specific activation of

all transmembrane proteins on the vascular membrane[Li and Xu (2007); Li and Xu (2000)], including receptors, ion channels, and ion pumps, and simultaneously activate multiple intracellular signaling pathways, such as MAPKs and PKCs, leading to pathophysiological changes of the cells, including inflammatory response, migration, proliferation, apoptosis and differentiation [Li and Xu (2007); Li and Xu (2000)]. Advanced glycation end product (AGE), oxidized low-density lipoproteins (oxLDL), and norepinephrine can respectively enable the additive activation of the intracellular signaling ERK phosphorylation induced by mechanical stress, resulting in increased proliferation of VSMCs [Zhang *et al.* (2013); Li *et al.* (2012); Liu *et al.* (2013)]. Recent studies have shown that mechanical stress can induce both proliferation and apoptosis of VSMCs and the more the two increases, the more obvious the vascular lesions are [Ping, *et al.* (2017); Ping *et al.* (2015)]. Correspondingly, the three members of MAPKs (ERK, JNK and p38MAPK) are selectively activated in the VSMCs [Ping *et al.* (2015)]. Among them, ERK is acknowledged to be associated with cell proliferation, while JNK and P38 are related to apoptosis. oxLDL specifically binds to its receptor LOX-1, enabling the superimposed promotion of the mechanical stress signal, promoting cell proliferation [Zhang *et al.* (2013)]. Macrophages are derived from the blood monocytes, and then migrate to the subendothelial layer after the damage of vascular endothelium. Stimulated by oxLDL, they can release many inflammatory factors and cytokines, and become foam cells via the phagocytosis of lipid, resulting in the formation of atherosclerotic plaques[Boyle (2005); Tuttolomondo *et al.* (2012); Justin Rucker and Crowley (2017); Dave, Ezhilan and Vasnawala et al. (2013); Tabas (2009)]. However, how are the macrophages affected by the mechanical stress in this process? Whether the mechanical stress and oxLDL alone or combined stimulation can cause the activation of MAPKs in the macrophages and lead to the simultaneous increase of cell proliferation and apoptosis? None of these have been reported yet.

Increasing data show that intracellular DNA promoter methylation can regulate gene expression, determine cell death and is directly related to vascular homeostasis [Xu *et al.* (2012); Ying *et al.* (2000); Kim *et al.* (2015)]. Ying *et al.* [Ying *et al.* (2000)] compared the SMCs of normal human aorta (representing the contractile phenotype) and those cultured in vitro (representing the in vivo synthetic phenotype), and they found that, in the contractile phenotype of SMCs, estrogen receptor (ER) promoter CpG island is unmethylated, but hypermethylated in the synthetic phenotype, suggesting ER methylated modification in the process of SMCs phenotypic change. However, in the process of mechanical-stress-induced apoptosis and proliferation of macrophages, whether the level of intracellular methylation changes, how such change is related to cell proliferation or apoptosis, neither of these has been reported.

Based on the previous work, in this study, we proposed that mechanical stress and oxLDL can be used alone or in combination to stimulate MAPK activation and the simultaneous increase in proliferation and apoptosis of macrophages, which are closely related to the methylation change. The results in this study will provide important and novel information on the pathogenesis of hypertension atherosclerosis.

2 Materials and methods

2.1 Cell culture and processing

The macrophages, RAW264.7 cells (ATCC, MD, U.S.), were cultured in high-dose-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, CA, U.S.) that was supplemented with 10% fetal calf serum (Gibco, Life Technologies, CA, U.S.) at 37 ℃ under 5% humidified CO_2. The subculture was carried out every 48 h after the cells had been carefully scrubbed.

Cells were seeded in silicone elastomer-bottomed six-well plates (Flexcell, Meckeesport, PA, U.S.) that were gelatin-coated (Sigma-Aldrich, St. Louis, MO, U.S.) at the proper density. After adherent growth, cells were then serum starved for 24 h and preprocessed with SIM (Sigma-Aldrich, U.S.) dissolved in ethanol for 1 h, and then ox-LDL and/or periodic stress (Flexcell, FX3000AFC-CTL) were applied as reported by Banes et al. [Banes, Gilbert and Taylor et al. (1985)]. Periodic stress is generated uniformly upon the cell membranes with periodic shape-changing (60 periods/min) and 10% extension of the plates.

2.2 Immunoblotting analysis

After pretreatment with SIM, MS (10% extension) and/or ox-LDL (50 μg/ml) was employed with the indicated time and concentration for the cells in the six-well plates. Lysis solution containing protease inhibitors was used to harvest cellular proteins. After heat denaturation and centrifugation of the lysate suspension, the concentration of proteins was evaluated with a Bio-Rad protein assay. Then, SDS-PAGE was used to separate the proteins. Electrophoresis was adopted to transfer them onto nitrocellulose filters. Detection, elution, and re-detection were carried out on these filters, with antibodies against p-ERKs(9101s), p-JNKs(9251s), p-p38MAPK(9211s), ERK(4695s), JNK (9258), p38MAPK(8690s) (Cell Signal.), and β-actin (Santa Cruz). Enhanced chemiluminescent indicators (GE Healthcare, Buckinghamshire, UK) were used to detect the binding antibodies, and the detection system (Image Quant LAS 4000 mini, GE Healthcare, Buckinghamshire, UK) was adopted for visualization. Image J software (national institutes of health) was adopted to analyze the results. Each experiment was performed independently at least thrice.

2.3 Reactive oxygen species measurement

After the cells were processed with MS and/or ox-LDL in the indicated pattern, time, and concentration, the cells were stained. We followed the manuals accompanying the fluorescent probe (H2DCFDA) (C400) (Life Technologies) and Hoechst 33342 (Sigma-Aldrich, U.S.) to show the reactive oxygen species (ROS) yield and the nucleus, respectively. Fluorescence microscopy (Olympus, Tokyo, Japan) was used to examine and obtain images of the cell. Accordingly, the samples were browsed in a zigzag course, and a representative spot and four regions around it from different directions at random were selected to take photographs. ROS positive (green staining) was adopted to identify the ROS yield. The proportion of ROS-positive cells represented the ROS rate. Each experiment was performed independently at least thrice.

2.4 Detection of proliferation, apoptosis, and resting cells in situ

Immunofluorescent staining with the Ki67 antibody (Santa Cruz) and the TdT-mediated dUTP nick end labeling (TUNEL) kit (Roche, Basel, Switzerland) coupled with 4′,6-diamidino-2-phenylindole (DAPI) were employed to examine the proliferative and apoptotic cells in situ. In short, the cells were treated with MS and/or ox-LDL or SIM for the indicated conditions. Then, the medium was removed and the pre-cooled methanol was added to fix the cells for 20 min in a refrigerator at −20 °C. We sealed the cells with 5% BSA; the primary antibody Ki67 (1:200) was incubated with the cells overnight at 4 ℃, and the corresponding CY3-conjugated secondary antibody (Jackson Immuno Research, U.S.) was incubated in a water-bath kettle for 2 h. The TUNEL kit was implemented following the instructions. DAPI was used to dye the nucleus for 10 min. Ki67-positive (red) and TUNEL-positive (green) staining were employed to identify the proliferation and apoptosis. The proportions of active proliferative or apoptotic cells were represented the proliferation rate or apoptosis rate, respectively. We regarded all of the remaining cells (DAPI stained only) as resting ones, with the exception of proliferating cells and those undergoing apoptosis. Each experiment was performed independently at least thrice.

2.5 Detection of genome-wide DNA methylation in situ

We used 3% paraformaldehyde to fix the processed cells. The 5-methylcytosine (5-mc, 1:200) antibody (BI-MECY-0100) (Eurogen), and CY3-conjugated secondary antibody (Jackson Immuno Research, U.S.) were used to incubate the cells. DAPI was used to dye the nucleus. We adopted 5-mc-positive staining (red) to identify the cells of DNA methylation, and the proportion of the 5-mc-positive cells among the total cell count represented the DNA methylation rate. Fluorescence microscopy (Olympus, Tokyo, Japan) was used to examine and obtain images of the cells. Each experiment was performed independently at least thrice.

2.6 Detection of genome-wide DNA methylation and proliferation in situ

The processed cells were fixed with 3% paraformaldehyde and incubated with 5-mc antibody and Ki67 antibody, with the corresponding CY3-conjugated secondary antibody and FITC-conjugated secondary antibody, which were employed to stain the cells. DAPI was used to dye the nucleus. We adopted 5-mc-positive staining (red) to identify the cells of DNA methylation, whereas Ki67-positive (green) staining was adopted to identify the proliferation of cells. The proportion of the 5-mc-positive cells among the total cell count represented the 5-mc positive rates, and ki67-positive cells among the total cell count represented the ki67-positive rates. Fluorescence microscopy (Olympus, Tokyo, Japan) was used to examine and obtain images of the cells. Each experiment was performed independently at least thrice.

2.7 Detection of genome-wide DNA methylation and apoptosis in situ

Similar to the previous processing method, the processed cells were fixed and treated with mannitol, and then the 5-mc antibody and the corresponding CY3-conjugated secondary antibody were employed to stain the cells. Meanwhile, the TUNEL kit was implemented following the manufacturer’s instructions. DAPI was used to dye the

nucleus, and TUNEL-positive (green) staining was adopted to identify the apoptotic cells. The proportion of the 5mc-positive cells among the total cell count represented the 5-mc positive rates, and the proportion of TUNEL- positive cells among the total cell count represented TUNEL-positive rates. Fluorescence microscopy (Olympus, Tokyo, Japan) was used to examine and obtain images of the cells. Each experiment was performed independently at least thrice.

2.8 Statistical analysis

As for the calculation of the percentage of positive-staining cells, the ImageJ program was used. The total number of cells was counted with DAPI (Hoechst33342)-stained cells, and the positive-staining cells were also counted. Then, the percentage was calculated. SPSS 20.0 (SPSS) was employed to carry out all analyses. Continuous variables are denoted as mean ± SEM, and categorical variables are denoted as measured numbers and percentages. ANOVA was performed on continuous variables and chi-square, whereas Fisher exact tests were performed on categorical variables. $P<0.05$ was referred to as statistically significant.

3 Results

3.1 MS and ox-LDL promote the simultaneous increases in proliferation and apoptosis of RAW 264.7 macrophages in an additive manner

MS promotes the proliferation and apoptosis of the VSMCs [Ping, *et al.* (2017); Zhang *et al.* (2013); Ping *et al.* (2015)], which are important cells for AS. To investigate the effect of MS on the proliferation and apoptosis of macrophages, the RAW 264.7 cells cultured on resting medium were processed by MS generated with Flexcell FX3000AFC-CTL. Then, cell proliferation and apoptosis were determined. As shown in Fig. 1A, and C, in comparison with the negative group, proliferation (Ki67-positive, red) and apoptosis (TUNEL-positive, green) of RAW 264.7 cells were increased by MS at the same time.

Ox-LDL is a key risk factor for AS that can result in proliferation and apoptosis of VSMCs or macrophages. To determine if MS-mediated proliferation and apoptosis can promote in an additive manner the ox-LDL effect, we followed Ki67 and TUNEL of the RAW 264.7 cells after treatment with ox-LDL (50 μg/ml) or MS plus ox-LDL. As reported, the proliferation and apoptosis of the RAW 264.7 cells were increased by the ox-LDL treatment [Yao *et al.* (2016)] (Fig. 1B). The combination treatment with MS and ox-LDL showed a significant upregulation compared with those of the single treatment (Fig. 1D), thereby suggesting an additive effect on the proliferation (Fig.1I) and apoptosis (Fig. 1J). Many resting cells (only blue) are also noted.

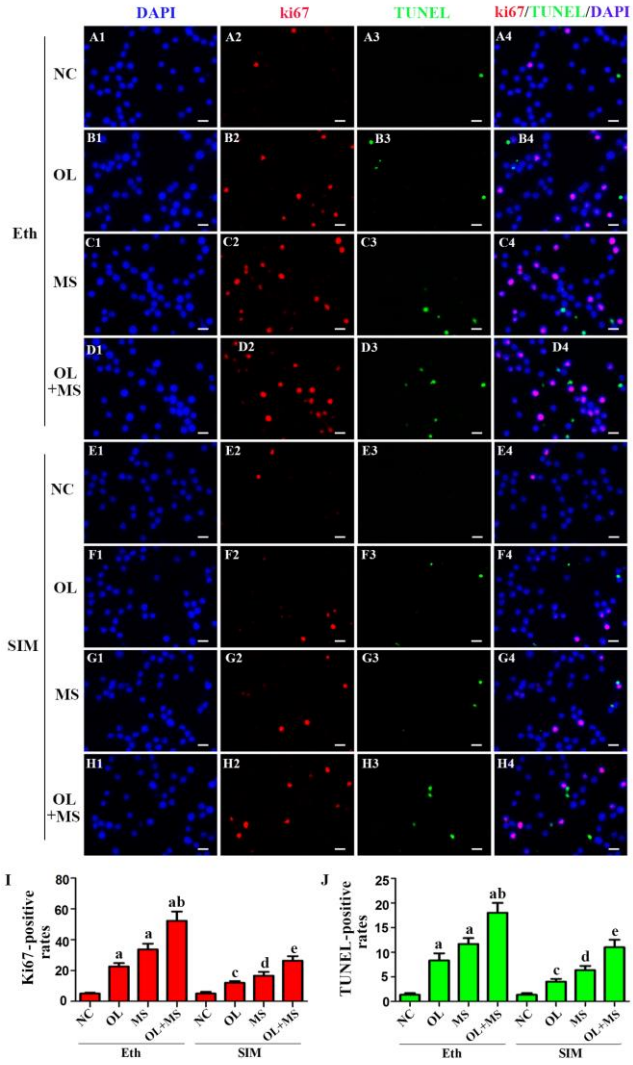

Figure 1: SIM relieved the effects induced by MS and ox-LDL on the proliferation and apoptosis of RAW 264.7 macrophages. RAW 264.7 cells cultured on resting medium were preprocessed by SIM for 1 h or not, processed by MS and/or ox-LDL(OL) for 1 h, and then continued incubating for another 23 h. In comparison with the NC (A), ox-LDL (B), or MS (C) caused the increase of proliferation (Ki67 positive, red) and apoptosis (TUNEL positive, green). Moreover, the combined stimulation (D) delivered anadditive effect. In comparison with the NC, (E) SIM had no effect on proliferation or apoptosis, whereas SIM relieved the effects induced by MS and/or ox-LDL on proliferation and apoptosis (F–H). The percentages of the Ki67-positive rate (I) and TUNEL-positive rate (J) were showed with the indicated treatments. SIM dissolved in ethanol (Eth). *Scale bar* represents 50 μm. All experiments were independently repeated thrice. a denotes $p < 0.05$ in respect to the NC group; b denotes $p < 0.05$ in respect to the OL or MS group; c denotes $p < 0.05$ in respect to OL; d denotes $p < 0.05$ in respect to MS; and e denotes $p <$

0.05 in respect to the combination of OL and MS.

3.2 SIM inhibits the proliferation and apoptosis induced by MS or/and ox-LDL

Statins are more effective than other lipid-regulating drugs at lowering the concentration of low-density lipoprotein (LDL) cholesterol. SIM is an effective lipid-lowering drug that can decrease LDL levels. It is primarily used to treat dyslipidemia and to prevent AS-related complications, such as stroke and heart attacks in those who are at high risk. It is now recommended for use along with a low-cholesterol diet. SIM has important effects on the status and function of macrophages; however, its influence on the proliferation and apoptosis caused by MS is unclear. In this study, RAW 264.7 cells were pre-processed by SIM (2.0 μM) for 1 h, then ox-LDL (50 μg/ml) or/and MS (10% stretch strength) were employed for another hour, and the culture was continued for 23 h. As shown, SIM had no effect on the proliferation and apoptosis of the RAW 264.7 cells compared with the negative control (Fig. 1A, E), whereas it partially inhibited the proliferation and apoptosis induced by ox-LDL (50 μg/ml) or/and MS (Figs. 1B–D, F–J). These data suggest that SIM can slow down the pathophysiological effects triggered by the upregulated proliferation and apoptosis of macrophages.

3.3 SIM depresses the phosphorylation of MAPKs induced by MS and/or ox-LDL

MAPK signal transduction pathways exist in most cells and mediate cellular responses to a diverse range of stimuli, which include growth factors, irradiation, and ox-LDL. We reported that MS promotes the proliferation and apoptosis of VSMCs by activating ERK1/2, JNKs, and p38MAPK [Ping *et al.* (2015)]. RAW 264.7 cells were processed and assessed by Western blot to investigate the effect of MS or/and ox-LDL on the MAPK activities of macrophages. Similar to VSMCs, ox-LDL upregulated the phosphorylation levels of the MAPK family (ERK1/2, JNKs, and p38MAPK) in a dose-dependent manner in RAW 264.7 cells, and ox-LDL was significantly higher than nLDL in the same dose (Figs.2A, C). However, phosphorylation levels of the MAPKs showed a biphasic expression pattern with the treatment of 50 μg/ml ox-LDL (Figs.2B, D). The MAPK activities were upregulated to the top in 10 min by processing with 10% extension MS (Figs.2E, G), and anadditive upregulation wasshown in the combined treatment with MS and ox-LDL (Figs.2F, H). ERK1 and ERK2 play an important role in the control of cell growth and differentiation, whereas JNKs and p38MAPK are related to apoptosis. The proliferation and apoptosis induced by MS might depend on the changes of MAPK phosphorylation levels in the RAW 264.7 cells.

To uncover the molecular mechanisms underlying the way by which SIM relieves the proliferation or apoptosis of RAW 264.7 macrophages induced by MS or/and ox-LDL, the MAPK activities were tested. SIM not only decreased the phosphorylation levels of MAPKs induced by MS in a dose-dependent manner (Figs.3A, B), but it also (SIM at 2.0 μM) depressed the phosphorylation levels of MAPKs induced by ox-LDL or the combination of ox-LDL and MS (Figs.3C, D). SIM might rely on the inhibition of phosphorylation levels of MAPKs induced by MS or/and ox-LDL in relieving proliferation and apoptosis.

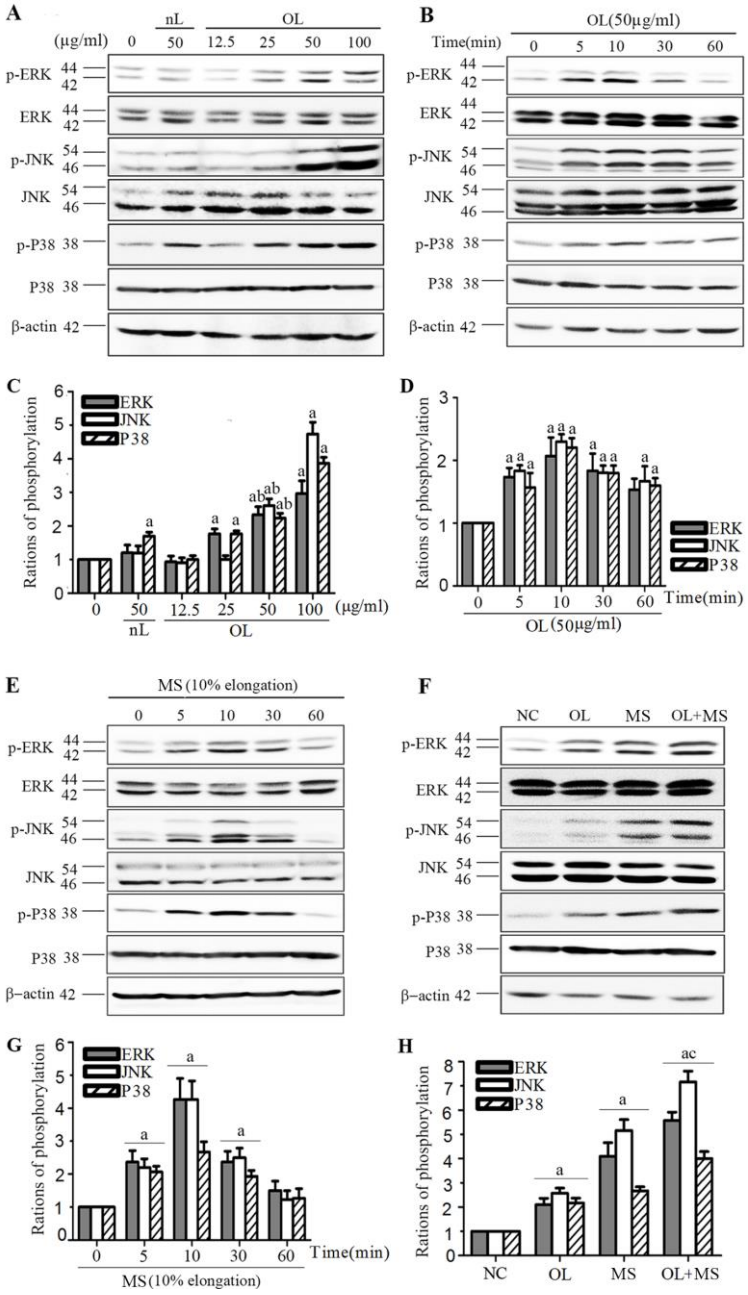

Figure 2: Phosphorylation levels of MAPKs induced by MS and/or ox-LDL in RAW 264.7 macrophages. RAW 264.7 cells were treated with ox-LDL or nLDL for 10 min in the indicated concentration. Compared with the NC or nLDL, the MAPK phosphorylation levels showed a dose-dependent increase by ox-LDL (A and C) and a biphasic expression pattern by time (B and D). When treated with MS in the indicated conditions, in comparison with the NC, the MAPK phosphorylation levels treated with S showed a

biphasic expression pattern by time (E and G), whereas the combination of MS (10% extension) and ox-LDL (50 μg/ml) showed an additive inducement on the phosphorylation of MAPKs (F and H). Phosphorylated kinase levels were calculated by comparing with the total kinases. All experiments were independently repeated thrice. a denotes $p< 0.05$ in respect to the NC group; b denotes $p< 0.05$ in respect to nL (50 μg/ml, 10 min); and c denotes $p< 0.05$ in respect to the OL or MS group.

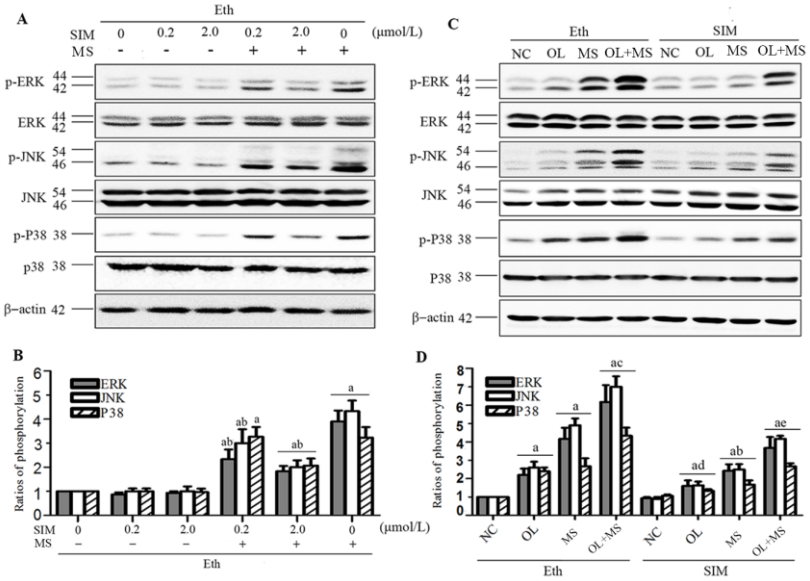

Figure 3: SIM depressed the phosphorylation levels of MARKs induced by MS and/or ox-LDL in RAW 264.7 macrophages. (A and B) Phosphorylation levels of MAPKs caused by MS in the cases with SIM (2.0 or 0.2 μM) or not; the statistical results of three independent trials. (C and D) Phosphorylation levels of MAPKs caused by MS and/or ox-LDL in the cases with SIM (2.0 μM) or not; the statistical results of three independent trials. a denotes $p< 0.05$ in respect to the NC group; b denotes $p< 0.05$ in respect to MS; c denotes $p< 0.05$ in respect to the OL or MS group; d denotes $p< 0.05$ in respect to OL; and e denotes $p< 0.05$ in respect to OL +MS.

3.4 SIM decreases DNA methylation induced by MS or/and ox-LDL

Numerous studies have shown that DNA methylation levels are associated with cell fate, i.e., cell proliferation, apoptosis, differentiation, early embryonic development, and the establishment of totipotency and pluripotency. The enhanced DNA methylation of the cystathionine γ-lyase (CSE) promoter exists in the ox-LDL-treated RAW 264.7 cells, which is associated with downregulated CSE transcription and the upregulated expression and activity of DNA methyltransferase (DNMT) [Du *et al.* (2016)]. However, we did not find a report on the relationship between DNA methylation and MS. To investigate the relationship between MS /ox-LDL and DNA methylation, RAW 264.7 cells were incubated with 5-mc antibody and CY3-conjugated secondary antibody in sequence and then checked with fluorescence microscopy. In this study, MS and ox-LDL upregulated DNA

methylation, and the additive effect was shown in the combination of MS and ox-LDL (Fig.4A–L, Y).

Furthermore, MS or/and ox-LDL upregulated DNA methylation levels with simultaneous increased cell proliferation, thereby showing that 5-mc was co-expressed with the majority of Ki67 in the same cells (Figs.5A–C). Furthermore, the combined administration delivered an additive effect (Fig.5D). Many reports have shown that DNA methylation and apoptosis have a close relationship. DNA methylation and apoptosis can be induced by MS or/and ox-LDL. Thus, we examined their relevance. The cells with upregulated DNA methylation did not overlap with the apoptotic ones, thereby suggesting a different mechanism related to apoptosis (Figs.6A-D). The upregulated DNA methylation induced by MS or/and ox-LDL was associated with proliferation but not apoptosis.

Statins are associated with demethylation by acting as DNMT inhibitors [Kodach *et al.* (2011)]. Lovastatin has downregulated DNMT activity, leading to promoter demethylation and upregulated expression of BMP2 and other genes methylated in colorectal cancer (CRC) [Kodach *et al.* (2011)] whereas SIM upregulated the Foxp3 level through the Foxp3 promoter demethylated in T-cells and induced BMP2 expression in the CRC xenograft mouse model [Kodach *et al.* (2011); Kim, Kim and Shevach (2010)]. In addition, SIM blocked the expressions and activities of MMP9, RhoA, and ROCK1 induced by AGE-BSA, of which the CpG site of the cis-promoter sequences was demethylated in keratinocyte [Lu *et al.* (2015)]. These data show the selectivity of SIM on DNA methylation regulation.

Whether SIM can regulate the DNA methylation levels induced by MS or/and ox-LDL in macrophages is unclear. As shown, SIM partially inhibited the upregulated DNA methylation induced by MS or/and ox-LDL (Figs.4M–X, Y), as well as simultaneously inhibited the increased cell proliferation. Furthermore, the Ki67 overlapped the 5-mc showing that the proliferating cells were the DNA methylated cells (Fig.5K). This finding suggested that the DNA methylation mediates the combined action of MS and ox-LDL in promoting the proliferation of macrophages (Figs.5E–H). Interestingly, SIM relieved the increased cell apoptosis induced by MS or/and ox-LDL; however, the TUNEL did not overlap the 5-mc (Figs, 6E–H). This finding indicated that the signaling of the DNA methylation and the apoptosis should be different. The inhibition of SIM on the methylation induced by MS and/or ox-LDL was associated with cell proliferation rather than apoptosis.

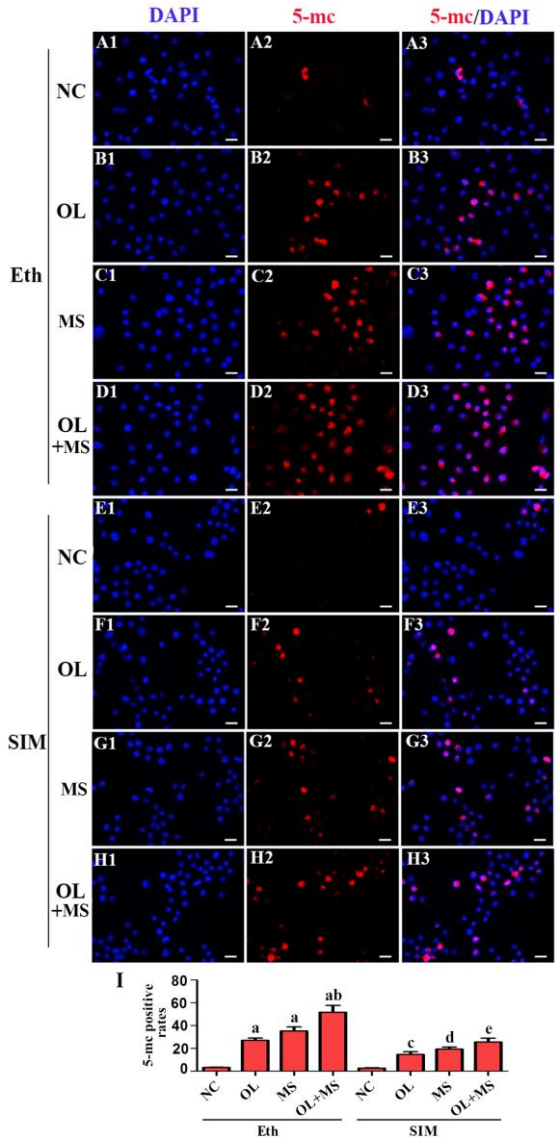

Figure 4: SIM downregulated DNA methylation caused by MS and/or ox-LDL in RAW 264.7 macrophages. After preprocessing with SIM (2.0 μM) for 1 h, RAW 264.7 cells were processed with ox-LDL and/or MS for another hour, and the normal culture lasted for 23 h. In comparison with the NC (A), SIM did not have an effect on the DNA methylation (5-mc-positive, red) (E), whereas MS (C) or ox-LDL (B) caused the increase of DNA methylation, which was depressed by SIM (F-G). At the same time, the additive effect (D) induced by the combination of MS and ox-LDL was also downregulated by SIM (H). Statistical graph of DNA methylation-positive rates obtained by three independent trials (I). a denotes $p< 0.05$ in respect to the NC group; b denotes $p< 0.05$ in respect to the OL or S group; c denotes $p< 0.05$ in respect to OL; d denotes $p< 0.05$ in respect to MS; and e denotes $p< 0.05$ in respect to the combination of OL and MS.

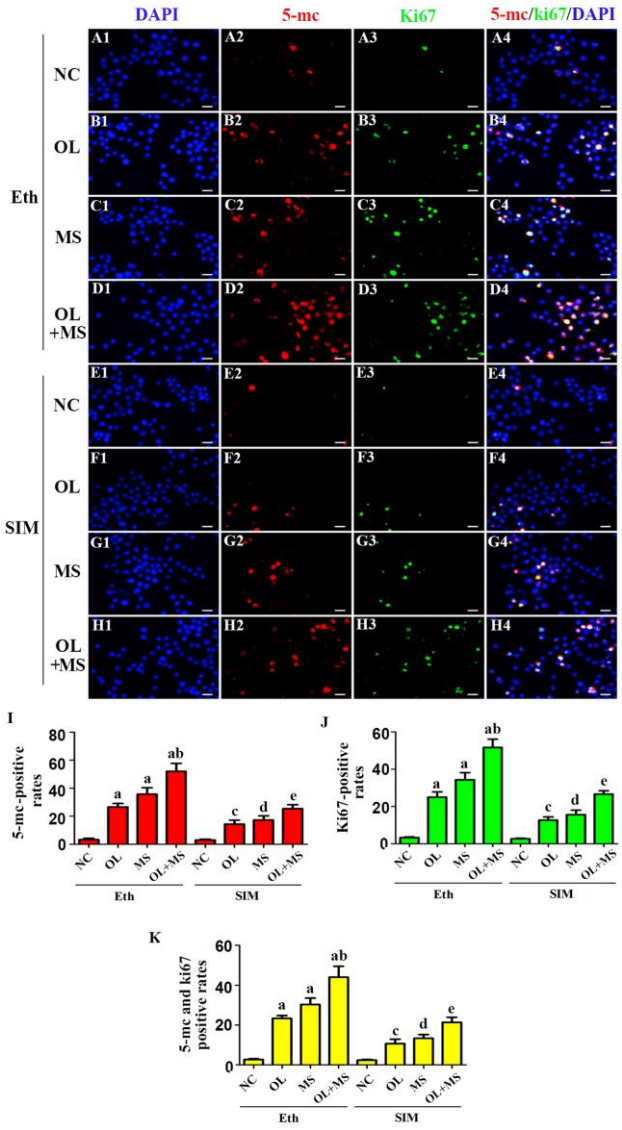

Figure 5: The relationship between DNA methylation and proliferation caused by MS and/or ox-LDL in RAW 264.7 macrophages. After preprocessing with SIM (2.0 μM) for 1 h, RAW 264.7 cells were processed with ox-LDL and/or MS for another hour, and the normal culture lasted for 23h. Genome-wide DNA methylation (5-mc-positive, red) and proliferation (Ki67-positive, green) overlapped each other from the observation of these merged pictures. In the NC, low 5-mc-positive cells were accompanied by a few Ki67-positive cells, and they were the same cells (A). In comparison with the NC, SIM did not have an effect on DNA methylation and proliferation (E), whereas MS (C) or ox-LDL (B) caused the increase of red and green in the same cells, which were depressed by SIM (F and G). At the same time, the additive effect (D) induced by the combination of MS and ox-LDL was also downregulated by SIM (H). Statistical graphs of

5-mcpositive rates and/or ki67-positive rates obtained by three independent trials (I and J). Scale bar represents 50 μm. a denotes $p< 0.05$ in respect to the NC group; b denotes $p< 0.05$ in respect to the OL or MS group; c denotes $p< 0.05$ in respect to OL; d denotes $p< 0.05$ in respect to MS; and e denotes $p< 0.05$ in respect to the combination of OL and MS.

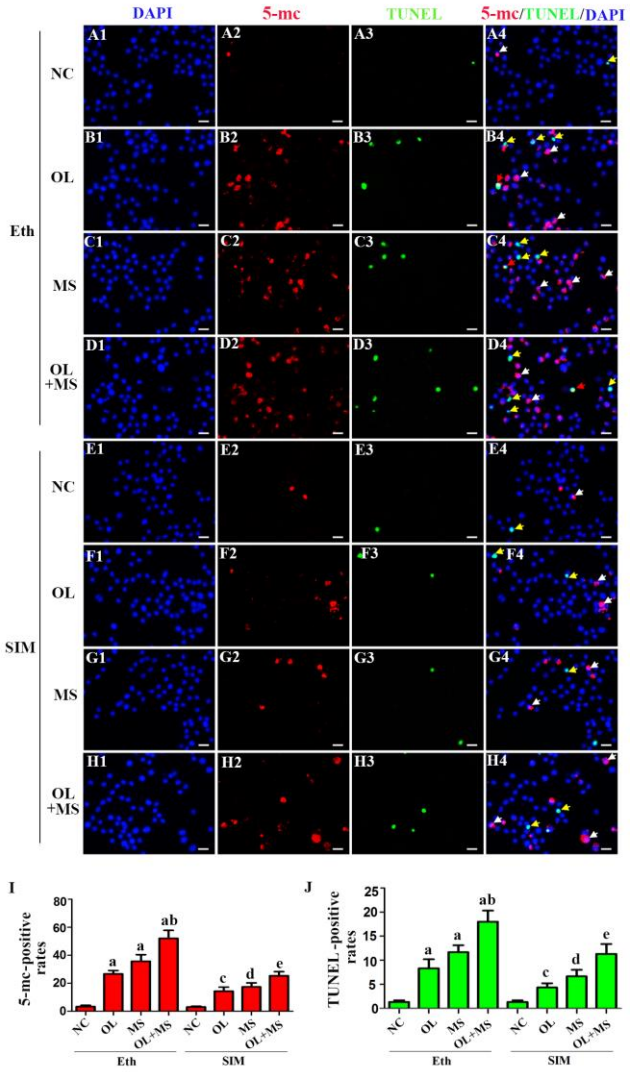

Figure 6: The relationship between the DNA methylation and apoptosis caused by MS and/or ox-LDL in RAW 264.7 macrophages. After preprocessing with SIM (2.0 μM) for 1 h, RAW 264.7 cells were processed with ox-LDL and/or MS for another hour, and the normal culture lasted for 23 h. The distribution of the genome-wide DNA methylation (5-mc-positive, red) and apoptosis (TUNEL-positive, green) were randomly scattered from the observation of these merged pictures. In the NC, low 5-mc-positive and few TUNEL-positive cells are scattered on the pictures (A). In comparison with the NC, SIM did not have an effect on DNA methylation and apoptosis (E), whereas MS (C) or

ox-LDL (B) caused the increase of DNA methylation and apoptosis that were depressed by SIM (F and G). At the same time, the additive effect (D) induced by the combination of MS and ox-LDL was also downregulated by SIM (H). Statistical graph of DNA methylation-positive rates and apoptosis-positive rates obtained by three independent trials (I and J). In the merged images, white arrows denote the 5-mc-positive cells, yellow arrows denote the TUNEL-positive cells, red arrows denote the TUNEL- and 5-mc positive cells. Scale bar represents 50 μm. a denotes $p< 0.05$ in respect to the NC group; b denotes $p< 0.05$ in respect to the OL or MS group; c denotes $p< 0.05$ in respect to OL; d denotes $p< 0.05$ in respect to MS; and e denotes $p< 0.05$ in respect to the combination of OL and MS.

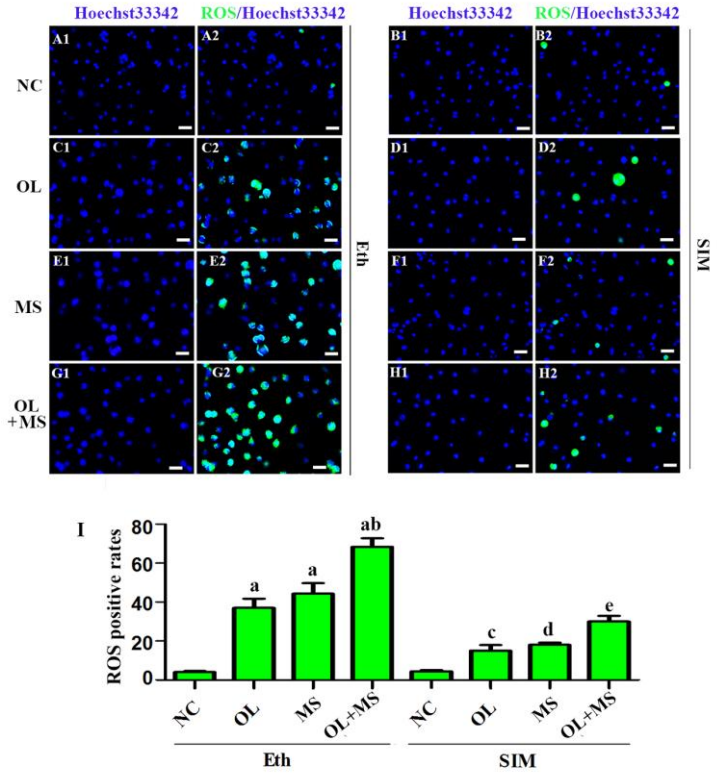

Figure 7: SIM depressed ROS yield induced by MS and ox-LDL in RAW 264.7 macrophages. RAW 264.7 cells cultured on resting medium were preprocessed by SIM for 1 h or not, processed by MS and/or ox-LDL for 10 min, and then stained with a fluorescent probe (H2DCFDA) (green) representing the ROS yield and Hoechst 33342 (blue) representing the nucleus. In comparison with the NC (A), SIM did not have an effect on ROS yield (B), whereas MS or ox-LDL had a promoting effect (C and E), which was depressed by SIM (D and F). At the same time, the additive effect induced by MS and ox-LDL was also decreased by SIM (G and H). (I) Statistical graph of ROS-positive rates obtained by three independent trials. Scale bar represents 50 μm. Following all versus the left preprocessed with Eth. a denotes $p< 0.05$, in respect to the NC group; b

denotes $p< 0.05$ in respect to the OL or MS group; c denotes $p< 0.05$ in respect to OL; d denotes $p< 0.05$ in respect to MS, and e denotes $p< 0.05$ in respect to the combination of OL and MS.

3.5 SIM depresses ROS yield induced by MS or/and ox-LDL

Endogenous ROS are natural byproducts of normal metabolism, and they play important roles in cell signaling and homeostasis [Devasagayam *et al.* (2004)], whereas cell dysfunction or death may happen with dramatically raised levels of ROS due to suffering from environmental stress (e.g. UV or ox-LDL) [Devasagayam *et al.* (2004); Li *et al.* (2016)].

To investigate the effect of MS on ROS generation in macrophages, RAW 264.7 cells were processed by MS and the ROS yield was monitored using a fluorescent probe (H2DCFDA). Consistent with the change of the phosphorylation of MAPKs, MS (10% stretch strength) or ox-LDL (50 μg/ml) provoked a significant increase in ROS generation in comparison with the negative control (Fig.7A, B, C, I). Moreover, a combination of MS and ox-LDL showed an additive effect (Fig.7G, I). Now that SIM can effectively decrease LDL levels, Fig.7D and I show a decrease of ROS by SIM which may be due to lowered ox-LDL. Nevertheless, SIM depressed the generation of ROS induced by MS or the combination of MS and ox-LDL in macrophages (Figs.7F, H, and I), which suggested that common pathways are found in the generation of ROS induced by MS and ox-LDL.

4 Discussion

Recently, we established a new approach to observe that the proliferation and apoptosis of VSMCs cultured on resting medium could be simultaneously increased by stimulation of MS and AGEs, and that the phosphorylation of the 3 members of MAPKs could also be increased[Ping *et al.* (2015); Ping *et al.* (2015); Li *et al.* (2012)]. In this study, we observed the effects of MS or oxLDL alone or in combination on the phosphorylation of ERK1/2, JNK1/2 and p38MAPKs, proliferation and apoptosis, and genome-wide DNA methylation in RAW264.7 macrophage, another important source of foam cells, cultured on resting medium, and explored the impacts of simvastatin on these effects. Several important novel findings were obtained as follows: (1) MS or oxLDL alone or in combination could promote the phosphorylation of ERK1/2, JNK1/2 and p38MAPKs in the macrophages cultured on resting medium, and the combined stimulation delivered a additive effect, while simvastatin could inhibit these effects; (2) MS or oxLDL alone or in combination could increase ROS yield in macrophages cultured on resting medium, and the combined stimulation delivered a additive effect, while simvastatin could inhibit these effects; (3) MS or oxLDL alone or in combination could simultaneously increase the proliferation and apoptosis in macrophages cultured on resting medium, and the combined stimulation delivered an additive effect, while simvastatin could inhibit these effects; (4) MS or oxLDL alone or in combination could increase methylation in macrophages cultured on resting medium, and the combined stimulation delivered a additive effect, while simvastatin could inhibit these effects; the fact that MS or oxLDL alone or in combination could increase methylation in macrophages cultured on resting

medium was associated with proliferation but not associated with apoptosis. In summary, this study for the first time revealed the pathophysiological effects of simulation by MS or oxLDL alone or in combination on the macrophages, expanded our current knowledge of the hypertension mechanical stress during the progression of atherosclerosis, and provided information for further investigating the pathogenesis and new prevention and treatment strategies of hypertension atherosclerosis.

The early stage of hypertension is characterized by arteriolosclerosis, accompanied by the hypertrophy or proliferation of medial smooth muscle, and its middle and late stages are characterized by large and medium arterial sclerosis or atherosclerosis, especially in concurrent hyperlipidemia. In hypertension, MS caused endothelium oxidative stress, leading to endothelial injury and lipid deposition; meanwhile, a variety of cell adhesion molecules and inflammatory factors are abnormally expressed in the endothelium, adsorbing the mononuclear cells in the blood; through the endothelial cells into the subendothelial layer, they are differentiated into the macrophages [Boyle (2005); Tuttolomondo *et al.* (2012); Justin Rucker and Crowley (2017); Vaillancourt, Ruffenach and Meloche et al. (2015); Stoneman and Bennett (2004)]. Under the increased mechanical stress, the phenotype of the endothelial SMCs changes and migrates into the subendothelial layer, growing along with the macrophages, releasing the inflammatory factors, and engulfing lipids, to become the foam cells [Tuttolomondo *et al.* (2012); Stoneman and Bennett (2004)]. The foam cells release lipid droplets during apoptosis, accelerating the formation of atherosclerotic plaques and promoting the progression of vascular remodeling and hypertension [Stoneman and Bennett (2004)]. This suggests that the functional changes of macrophages are closely related to the formation of hypertension. However, once hypertension is formed, the mechanical stress generated by higher blood pressure will directly act on all the cells in the vessel walls, including the endothelial cells (tunica interna), SMCs (tunica media), fibroblasts, and undifferentiated mesenchymal stem cells (tunica externa), thereby changing the structure and function of vascular cells and tissues and accelerating the vascular disease [Li and Xu (2007); Li and Xu (2000)]. A lot of studies have been made to report the impacts of shear stress on the endothelial cells, those of stress on VSMCs, and those of oxLDL on the structure and function of macrophages and VSMCs. However, the influences of the mechanical stress or oxLDL alone or in combination on the macrophages are rarely reported. In this study, we first reported that MS or oxLDL alone or in combination could increase the phosphorylation of MAPKs, oxLDL could induce the activation of MAPKs in a time- and concentration-dependent manner, MS alone could also induce the activation of MAPKs, and the combined stimulation could deliver an additive activation. MS or oxLDL alone or in combination could induce the increase of both proliferation and apoptosis, and the combined stimulation could deliver an additive activation. At the same time, simvastatin could be inhibited the 3 members of of MAPKs simultaneously activated by MS or oxLDL alone or in combination, so as to inhibit the simultaneous increase of proliferation and apoptosis. This further emphasized the close association between the MAPK signaling and proliferation and apoptosis, as well as the role of simvastatin in preventing atherosclerosis.

MAPKs are the most important signal transduction molecules in cells, involved in essential cell events including differentiation, migration, inflammation, proliferation, and

apoptosis (11,12). It has been commonly recognized that the phosphorylation of ERKs is closely related to proliferation, and the activation of JNKs and p38MAPK is related to apoptosis. Many papers have reported that oxLDL induces the activation of the 3 members of MAPKs in macrophages [Deigner and Claus (1996); Yin, Liu and Ji et al. (2013); Wang *et al.* (2014); Shao *et al.* (2012)]. oxLDL mainly induces the proliferation of macrophages via the signaling pathways of PI3K/Akt and MAPK/ERK [Yao *et al.* (2016)]. Low-dose oxLDL synergizes low-level bacterial lipopolysaccharide (LPS) via ERK pathway to promote the macrophages in secreting pro-inflammatory factors [Wiesner *et al.* (2010)], and inhibition of MAPKs could inhibit the inflammatory response, apoptosis and foam formation induced by the oxLDL in the macrophages [Wang *et al.* (2013)]. oxLDL can induce the activation of p38MAPK and the transcriptional activation of PPARγ (a nuclear receptor), promoting the expression of CD36 and ultimately the formation of foam cells. As far as we know, the effects of mechanical stress on the macrophages have not been reported yet, but its effects on VSMCs have been extensively studied. We had previously reported that MS or oxLDL alone or in combination could activate ERK1/2 in the VSMCs to promote proliferation[Zhang *et al.* (2013)]. More recently, we found that MS could activate both ERK and JNKs and p38MAPK in the VSMCs, and that the 3 members of MAPKs were selectively activated within the cells[Ping *et al.* (2015)]. In this study, oxLDL could activate MAPKs in the macrophages in a time- and concentration-dependent manner, while MS alone could also induce the activation of MAPKs, the combined stimulation could deliver a additive activation. These findings further underlined that the mechanical stress generated by higher blood pressure could activate the MAPKs signaling of the macrophages in the plaques, while the activation of MAPKs would definitely lead to changes in cell death and proliferation. It has been reported that oxLDL induces the activation of MAPKs, resulting in increased proliferation or apoptosis in macrophages [Shao, Han and Peng et al. (2016); Ishii *et al.* (2009); Tiwari, Singh and Barthwal (2008)]. Logically, these results seem to be contradictory. Why do the cells show their opposite fates of life and death under the same stimulation? Are these reported results actually existing or just experimental error between different groups? Since the cell proliferation and apoptosis have always been investigated separately, these questions still cannot be answered. Recently, we have established a new method that allows us to simultaneously observe the presence of proliferation, apoptosis, and resting cells in the same field of view, as well as the distribution of various cells. Using this technique, we found that the proliferation and apoptosis of VSMCs cultured on resting medium could be simultaneously increased by mechanical stress, with an additive effect of AGEs [Ping *et al.* (2015)]. In this study, we similarly found that mechanical stress could induce both proliferation and apoptosis in macrophages, while the combined stimulation by MS and oxLDL delivered an additive effect (Fig. 4 and 5). The results suggest that, after the same stimulation, proliferation and apoptosis simultaneously increased in cells is a universal biological phenomenon, which can be similarly found in both VSMCs and macrophages. The underlying mechanism behind this phenomenon deserves further study. For instance, it has been reported in the literature that the intracellular concentration of the reactive oxygen species (ROS) will rise after extracellular stimulation, and the increased level is closely related to the cell survival and death. Physiological concentration is necessary for the physiological

metabolism of cells, but a too high level may lead to cell death [Napoli, de Nigris and Palinski (2001); Dimmeler and Zeiher (2000); Ermak, Lacour and Drueke et al. (2008)]. In our experiments, it was found that stimulation by MS or oxLDL alone could increase ROS, while the combined stimulation delivered an additive effect. However, through further observation, we could also find a fact that the ROS level in each cell of the same group under the same conditions may vary, presenting as negative, weak positive, and strong positive (Fig.7). Therefore, this result was consistent with the simultaneous presence of cell proliferation and apoptosis. The simultaneous activation of the three members of MAPKs also indicated a simultaneous increase of proliferation and apoptosis (Figs.2 and 3).

DNA promoter methylation could regulate gene expression, determine cell survival and death, and is directly related to vascular homeostasis and tumorigenesis [Xu *et al.* (2012); Ying *et al.* (2000); Kim *et al.* (2015)]. DNA methylation refers to the process that, under the catalysis of DNA methylation transferase (DNMT), the activated methyl is introduced into a DNA chain to form 5-methylcytosine, with S-adenosylmethionine (SAM) as the methyl donor [Kim *et al.* (2015)]. In mammals, methylation mainly occurs on the cytosine of CpG dinucleotide sequence of the DNA molecule. The methylation of CpG is usually associated with gene silencing, directly regulating the cell proliferation and apoptosis [Kim *et al.* (2015)]. In order to further explore the mechanism of macrophages proliferation and apoptosis induced by MS or oxLDL alone or in combination, we investigated the relationship between DNA methylation and proliferation and apoptosis. We found that, compared with the negative group, stimulation by MS or oxLDL alone or in combination could cause the increase in the genome-wide DNA methylation in macrophages, and the combined stimulation of the two delivered an additive effect. The increase of DNA methylation is closely related to proliferation but not associated with apoptosis, because most cells of increased methylation share overlapped cellular signal with the cells of proliferation, but not with those of apoptosis (Figs.5 and 6). However, the reason why higher methylation is associated with proliferation remains unknown. The genes associated with proliferation (proto-oncogene) and proliferation inhibition (tumor suppressor gene) simultaneously exist in the cells. For the former, if high methylation occurs, then proliferation is inhibited and apoptosis is increased; for the latter, if high methylation occurs, then proliferation is increased and apoptosis is decreased, and vice versa [Fu *et al.* (2010)]. In addition, different cell phenotypes show different changes in DNA methylation under the same extracellular stimuli. Ying *et al*. [Ying *et al.* (2000)] found that the levels of CpG methylation of estrogen receptor (ER) promoter are different between the SMCs in normal human aorta (representing the contractile phenotype) and SMCs cultured *in vitro* (representing the *in vivo* synthetic phenotype), as the former was non-methylated, while the latter was highly methylated. This suggests that, in the process of SMCs phenotype change, the methylated modification of ER occurs; similarly, DNA promoter methylation is different in the genes between different subtypes of macrophages (M1 and M2), the functions of which are also different [Babu *et al.* (2015)].

Statins were first used to reduce blood lipids and to prevent the occurrence of hyperlipidemia atherosclerosis. Today, both the clinical and basic researches have shown that the role of statins is pleiotropic (24,25). In this study, we found that simvastatin could significantly inhibit the simultaneous increase of proliferation and apoptosis in

macrophages caused by MS or oxLDL alone or in combination, by reducing MAPKs activation, ROS yield, and genome-wide DNA methylation. The results suggest that simvastatin may play an important role in inhibiting the development of atherosclerosis induced by hypertension and hyperlipidemia.

In conclusion, this study for the first time found that MS or oxLDL alone or in combination could selectively induce the up-regulation of genome-wide DNA methylation via the MAPKs and oxidative stress signaling pathway in macrophages, to promote the simultaneous increase of proliferation and apoptosis in macrophages, while the combined use of the two delivered an additive effect; these effects could be inhibited by simvastatin. These data will further expand the knowledge of the molecular mechanisms of hypertension vascular remodeling, providing new strategies for prevention and treatment of vascular remodeling during hypertension complications, such as hyperlipidemia.

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