

Oleic acid induces A7r5 cell proliferation and migration associated with increased expression of HGF and p-p38

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Abstract. The phenotypes and mechanisms underlying the proliferation and migration of vascular smooth muscle cells (VSMCs) induced by oleic acid (OA) are not completely understood. Therefore, the aim of the present study was to further elucidate the effects of OA on the proliferation and migration of VSMCs. Using A7r5 cells, the hepatocyte growth factor (HGF) inhibitor PHA665752 and the p38 MAPK inhibitor SB203580 were utilized, and Cell Counting Kit-8 (CCK-8) assays, Transwell assays, flow cytometry, ELISAs, western blotting and reverse transcription-quantitative PCR (RT-qPCR) were conducted to assess the effects of OA. CCK-8 assays indicated that OA promoted (at 5 and 50 $\mu\text{mol/l}$) or inhibited (at 800 $\mu\text{mol/l}$) A7r5 cell proliferation in a time- and concentration-dependent manner ($P<0.05$). Transwell assays revealed that OA also promoted (at 50 $\mu\text{mol/l}$) or inhibited (at 800 $\mu\text{mol/l}$) A7r5 cell migration ($P<0.05$). Moreover, cell-cycle analysis identified that 50 $\mu\text{mol/l}$ OA reduced the cellular population in the G₀/G₁ phase and enhanced the cellular population in the S phase ($P<0.05$), whereas 800 $\mu\text{mol/l}$ OA increased the cell number in the G₀/G₁ phase and decreased the cell number in the S phase ($P<0.05$). In addition, OA promoted (at 50 $\mu\text{mol/l}$) or inhibited (at 800 $\mu\text{mol/l}$) the expression level of HGF in A7r5 cells, as demonstrated via ELISA, western

blotting and RT-qPCR analyses ($P<0.05$). It was also found that OA promoted (at 50 $\mu\text{mol/l}$) or inhibited (at 800 $\mu\text{mol/l}$) the expression level of phosphorylated (p)-p38 in A7r5 cells, as indicated by western blotting ($P<0.05$). Furthermore, the cell proliferation, migration and HGF expression induced by OA (50 $\mu\text{mol/l}$) were mitigated by treatment with PHA665752 (0.1 $\mu\text{mol/l}$) ($P<0.05$), and the cell proliferation, migration and p-p38 expression induced by OA (50 $\mu\text{mol/l}$) were mitigated by SB203580 (2 $\mu\text{mol/l}$) ($P<0.05$). Thus, the results suggested that OA served a role in the proliferation and migration of VSMCs via HGF and the p38 MAPK pathway. Moreover, the proliferation and migration of VSMCs induced by OA was associated with increased expression levels of HGF and p-p38. Taken together, OA, HGF and p38 MAPK may be potential therapeutic targets for the treatment of atherosclerosis.

Introduction

Hypertriglyceridemia (HTG) refers to serum triglyceride (TG) levels at ≥ 150 mg/dl or ≥ 1.7 mmol/l, and its prevalence increases with age (1). Previous studies have reported that HTG is one of the risk factors for numerous cardiovascular diseases (CVD) (2,3). Glycerin and fatty acids (FAs) are common metabolites of TGs, and serve a role in energy metabolism and maintaining physiological functions in multiple organisms (4,5). However, the effects and mechanisms of TGs or how their metabolites alter the structure and function of vascular smooth muscle cells (VSMCs) require further investigation.

The abnormal proliferation and migration of VSMCs is one of the characteristics of atherosclerosis (6-8). Zhou *et al* (9) revealed that high TG serum levels could stimulate the proliferation of human fetal VSMCs and affect the ultrastructure of cells, but results from Yin *et al* (10) suggested that high TG serum levels did not significantly promote the proliferation of VSMCs. Furthermore, Mattern and Hardin (11) reported that oleic acid (OA) could reduce the apoptosis of VSMCs induced by palmitic acid. Our previous studies indicated that triolein (12) and medium-chain TGs (13) can promote (at low concentrations) or inhibit (at high concentrations) the proliferation of rat aortic VSMCs in a time- and concentration-dependent manner.

OA, a monounsaturated FA, representing ~80% of plasma phospholipid monounsaturated FAs, is one of the primary FA

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Abbreviations: CCK-8, Cell Counting Kit-8; CVD, cardiovascular diseases; FAs, fatty acids; HGF, hepatocyte growth factor; HTG, hypertriglyceridemia; OA, oleic acid; p-p38, phosphorylated p38; RT-qPCR, reverse transcription-quantitative PCR; ROS, reactive oxygen species; TG, triglyceride; VSMCs, vascular smooth muscle cells

Key words: OA, VSMCs, proliferation, migration, HGF, p-p38

components of the Mediterranean diet and olive oil, is involved in the regulation of cell proliferation (14,15). Moreover, it is one of the metabolites derived from TGs (15). However, the effects and mechanisms via which OAs regulate cell proliferation remain to be elucidated.

Hepatocyte growth factor (HGF) is a cytokine with multiple biological effects, such as promoting cell survival and the regeneration of tissues, and suppressing and improving chronic inflammation and fibrosis (16), and serves a role in promoting or inhibiting cell proliferation (17). However, to the best of our knowledge, whether the HGF signaling pathway is involved in the OA-induced proliferation of VSMCs is largely unknown, and this issue was first discussed by Greene *et al.* (18).

MAPK is a key signaling pathway that regulates cell proliferation, apoptosis, inflammatory responses and differentiation by activating a series of downstream regulatory factors (19,20). The MAPK family includes the JNK and p38 families (21,22). Furthermore, p38 MAPK signaling is closely associated with atherosclerosis, as it has been demonstrated to serve a role in VSMC (23) and H441 cell proliferation (24), HGF secretion (25) and connexin 43 expression (26).

Therefore, the main aim of the present study was to elucidate the relationship between the effect of OA on VSMCs and the HGF and p38 MAPK-signaling pathways.

Materials and methods

Reagents. High glucose (25 mmol/l) DMEM was purchased from Gibco (Thermo Fisher Scientific, Inc.). OA was purchased from the Cayman Chemical Company. Cell Counting Kit-8 (CCK-8) assays were purchased from the Beyotime Institute of Biotechnology, while PI was purchased from Sigma-Aldrich (Merck KGaA). Commercial HGF ELISA kit (cat. no. EK1301) was purchased from Boster Biological Technology. RevertAid First Strand cDNA Synthesis kits were purchased from Hangzhou Woosen Biotechnology Co., Ltd., and SYBR Green quantitative PCR (qPCR) mix for reverse transcription-qPCR (RT-qPCR) was purchased from Takara Biotechnology Co., Ltd. Transwell chambers were obtained from Corning, Inc. Rabbit anti-human polyclonal antibody against HGF (cat. no. ab83760) was purchased from Abcam, p38 (cat. no. AM8123) and phosphorylated p38 (p-p38; cat. no. AM1195) antibodies were obtained from Hunan Auragene Biotechnology Co., Ltd., and monoclonal antibodies against GAPDH (cat. no. 8884) were purchased from Cell Signaling Technology, Inc. The PVDF membrane and ECL detection kits were obtained from EMD Millipore. Goat anti-rabbit (cat. no. SA009) and mouse (cat. no. SA001) IgG secondary antibodies conjugated to HRP, the BCA protein assay kit, DMSO, SDS-PAGE loading buffer, TRIzol[®] reagent and RIPA buffer were obtained from Hunan Auragene Biotechnology Co., Ltd. Penicillin and streptomycin were purchased from MP Biomedicals, LLC. The HGF inhibitor PHA665752 (27) and the p38 MAPK inhibitor SB203580 were obtained from Selleck Chemicals.

Cell line and culture. A7r5 cells, a smooth muscle cell line derived from the rat thoracic aorta, were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in high glucose

(25 mmol/l) DMEM supplemented with 10% FBS (Hunan Auragene Biotechnology Co., Ltd.) (28), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Cell proliferation assays. A7r5 cells were seeded in a 96-well tissue culture plate at a density 5x10³ cells/well. DMSO (50 mmol/l) was used as a solvent to dissolve OA. The final concentration of DMSO in all experimental groups was not >1 mmol/l (<0.1%) (29,30). Several studies have suggested that treatment with 1-25 μM SB203580 (31,32) and 0.1 μmol/l PHA665752 (30,33,34) does not induce cell death and does not reduce the number of cells cultured. Thus, these cells were then incubated at 37°C with different concentrations of OA (0, 0.5, 5, 50, 200 or 800 μmol/l) for 24 and 48 h, or 0 μmol/l OA, 50 μmol/l OA, 50 μmol/l OA+ 0.1 μmol/l PHA665752 or 50 μmol/l OA+ 2 μmol/l SB203580 for 24 h. Concentrations of 0.1 and 2.0 μmol/l represented the IC₅₀ of PHA665752 and SB203580, respectively. Moreover, 0 μmol/l OA (containing 1 mmol/l DMSO) served as a control. After treatment, the medium was removed from the wells, and 0.1 ml high glucose DMEM containing 10% CCK-8 was added to each well for 3 h at 37°C according to the manufacturer's instructions. Cell viability was determined by measuring the absorbance at 450 nm using averages from quintuplicate wells, and normalized to a control well. The absorbance at each time point was determined using a microplate reader. All experiments were performed ≥3 times.

Cell cycle analysis. A7r5 cells were seeded in a 6-well tissue culture plate at a density 2.5x10⁵ cells/well. These cells were then incubated at 37°C with three different concentrations of OA (0, 50 or 800 μmol/l) for 24 h; 0 μmol/l OA (containing 1 mmol/l DMSO) served as a control. After treatment, cells were harvested using trypsin. The cells were centrifuged at 300 x g for 5 min at 25°C, washed twice with cold PBS and fixed with 75% cool ethanol at -20°C overnight (not <16 h). The fixed cells were then centrifuged at 200 x g for 5 min at 25°C and washed twice with cold PBS. Then, cells were stained with 50 μg/ml PI containing 10 μg/ml RNase A for 30 min on ice. Finally, the distributions of cells in different cell cycle phases were assessed using specific amounts of cellular DNA (2x10⁵ cells/tube) and flow cytometry (CytoFLEX; Beckman Coulter, Inc.). In total, >10,000 cells were counted per sample, and DNA histograms for cell cycle analysis were analyzed using Cell Quest software version 2.0 (BD Biosciences). The percentage of cells in the G₀/G₁ phase, S phase and G₂/M phase were analyzed, and all experiments were repeated in triplicate.

Transwell assays. A7r5 cells in logarithmic growth phase were harvested with trypsin, and then starved in serum-free medium. Next, a cell suspension (2x10⁴ cells) in 0.3 ml high-glucose DMEM was seeded into the upper well of a Transwell chamber (0.33 cm² growth surface area; 8-μm pore size), and these cells were then treated at 37°C with three concentrations of OA (0, 50 or 800 μmol/l), or 0 μmol/l OA, 50 μmol/l OA, 50 μmol/l OA+ 0.1 μmol/l PHA665752 and 50 μmol/l OA+ 2 μmol/l SB203580 for 24 h. Moreover, 0 μmol/l OA (containing 1 mmol/l DMSO) served as a control. In the lower chamber, 0.5 ml high-glucose DMEM with 10% FBS was added. After

incubation for 24 h at 37°C in a humidified incubator with 5% CO₂, the cells on the upper side of the upper chamber were removed using a cotton swab. The lower side of the upper chamber was fixed with 3% methanol for 5 min at 25°C, and stained with 2% crystal violet for 10 min at 25°C. The number of cells penetrating across the membrane was counted using an inverted microscope at x100 magnification in three random visual fields, and all experiments were performed in triplicate.

HGF level analysis using ELISA. After stimulation at 37°C with three different concentrations of OA (0, 50 or 800 μmol/l) for 24 h, A7r5 cells (1x10⁶ cells) were collected and centrifuged at 1,000 x g for 10 min at 25°C to remove debris. The 0 μmol/l OA (containing 1 mmol/l DMSO) served as a control. HGF protein levels in these cells were quantified using an ELISA kit (Abcam), according to the manufacturer's instructions, and all samples were measured three times. All experiments were repeated ≥3 times.

Western blotting. After stimulation at 37°C with three different concentrations of OA (0, 50 or 800 μmol/l), or 0 μmol/l OA, 50 μmol/l OA, 50 μmol/l OA+ 0.1 μmol/l PHA665752 and 50 μmol/l OA+ 2 μmol/l SB203580 for 24 h, cells (1x10⁶ cells) were washed with PBS and then lysed in RIPA buffer for 30 min at 4°C. The 0 μmol/l OA (containing 1 mmol/l DMSO) served as a control. The total protein concentrations were measured using a BCA protein assay kit. Equal amounts of protein (20 μg/well) were then mixed with SDS-PAGE loading buffer and boiled for 10 min. These samples were separated using 12% SDS-PAGE, and then transferred to a PVDF membrane. Membranes were incubated with appropriate concentration of primary antibody for HGF (1:100 dilution), p38 (1:1,000 dilution), p-p38 (1:1,000 dilution) or GAPDH (1:1,000 dilution) overnight at 4°C. After washing three times with 1X TBS-0.1% Tween 20, these membranes were incubated for 1 h with goat anti-rabbit or anti-mouse IgG secondary antibodies (1:15,000 dilution) conjugated to HRP for evaluating the expression levels of HGF and GAPDH. Proteins were visualized using an ECL detection kit, and the relative band intensities were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Inc.). All results were verified using ≥3 independent experiments.

RNA isolation, RT and RT-qPCR. Total RNA was isolated from A7r5 cells (1x10⁶ cells), after stimulation at 37°C with varying concentrations of OA (0 μmol/l OA, 50 μmol/l OA and 50 μmol/l OA+ 0.1 μmol/l PHA665752) for 24 h, using TRIzol reagent, according to the manufacturer's instructions. The 0 μmol/l OA (containing 1 mmol/l DMSO) served as a control. cDNAs were synthesized using 1 μg total RNA with the RevertAid First Strand cDNA Synthesis kit, following the manufacturer's instructions. RT-qPCR was performed using SYBR Green qPCR mix, following the manufacturer's instructions. The β-actin gene was measured as an internal quantitative control. All reactions were carried out on an ABI® 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) in duplicate. The reaction conditions were as follows: Initial denaturation at 95°C for 3 min; followed by 40 cycles of denaturation at 95°C for 10 sec, annealing and elongation at 60°C for 10 sec; and final extension at 72°C for 30 sec. Melt curves were analyzed for each sample. The

average value in each duplicate was used to calculate the relative amount of HGF using the 2^{-ΔΔC_q} method (35). The primer sequences were as follows: HGF forward, 5'-TCATTGGTA AAGGAGGCA-3' and reverse, 5'-GTCACAGACTTCGTA GCG-3'; and β-actin forward, 5'-AGGCCCTCTGAACC CTAAG-3' and reverse, 5'-CCAGAGGCATACAGGGAC AAC-3'. All experiments were conducted ≥3 times.

Statistical analysis. Data are presented as the mean ± SD. All statistical analyses were performed using SPSS 20.0 software (IBM Corp.). Differences between two groups were compared using a two-tailed Student's t-test, and the one-way ANOVA followed by Tukey's test was performed to compare data between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of OA on A7r5 cell proliferation and migration. First, A7r5 cells were treated with increasing concentrations of OA (0, 0.5, 5, 50, 200 and 800 μmol/l). Then, CCK-8 assays were conducted to evaluate the cell viability at two time points (24 and 48 h post-treatment), based on findings from previous studies (12,13). The current results demonstrated that OA treatment promoted A7r5 cell proliferation at lower concentrations (5 and 50 μmol/l), but inhibited A7r5 cell proliferation at higher concentrations (800 μmol/l), in a time- and concentration-dependent manner (Fig. 1A). The optimal parameters for promoting cell proliferation were determined to be 24 h and a dose of 50 μmol/l OA. According to the aforementioned results, three concentrations of OA (0, 50 and 800 μmol/l) and the treatment period of 24 h, were selected for subsequent experiments. Treatment with 0 μmol/l OA (containing 1 mmol/l DMSO) served as a control group.

Next, A7r5 cells were incubated with three different OA conditions (0 μmol/l OA, 50 μmol/l OA or 50 μmol/l + 0.1 μmol/l PHA665752) for 24 h. The data indicated that the effect of OA (at 50 μmol/l) in promoting cell proliferation was mitigated by PHA665752 (0.1 μmol/l) (Fig. 1B). Subsequently, A7r5 cells were treated with three different concentrations of OA (0, 50 and 800 μmol/l) for 24 h. Compared with the 0 μmol/l OA group (G₀/G₁ phase, 65.72±1%; S phase, 25.31±0.3%), the results of flow cytometry demonstrated that low concentrations of OA (at 50 μmol/l) reduced the number of cells in the G₀/G₁ phase (56.06±1.6%) and increased the number of cells in the S phase (33.71±0.9%), while high concentrations of OA (at 800 μmol/l) increased the number of cells in the G₀/G₁ phase (73.64±1.1%) and decreased the number of cells in the S phase (16.52±0.7%) (Fig. 1C).

Transwell assay results indicated that low concentrations of OA (at 50 μmol/l) promoted cell migration, but high concentrations of OA (at 800 μmol/l) suppressed cell migration (Fig. 1D). Additionally, when A7r5 cells were incubated with 0 μmol/l OA, 50 μmol/l OA or 50 μmol/l OA +0.1 μmol/l PHA665752 for 24 h, the results suggested that the effect of OA (at 50 μmol/l) in promoting cell migration was mitigated by treatment with PHA665752 (0.1 μmol/l) (Fig. 1E).

Effects of OA on HGF expression in A7r5 cells. OA treatment increased HGF expression at low concentrations

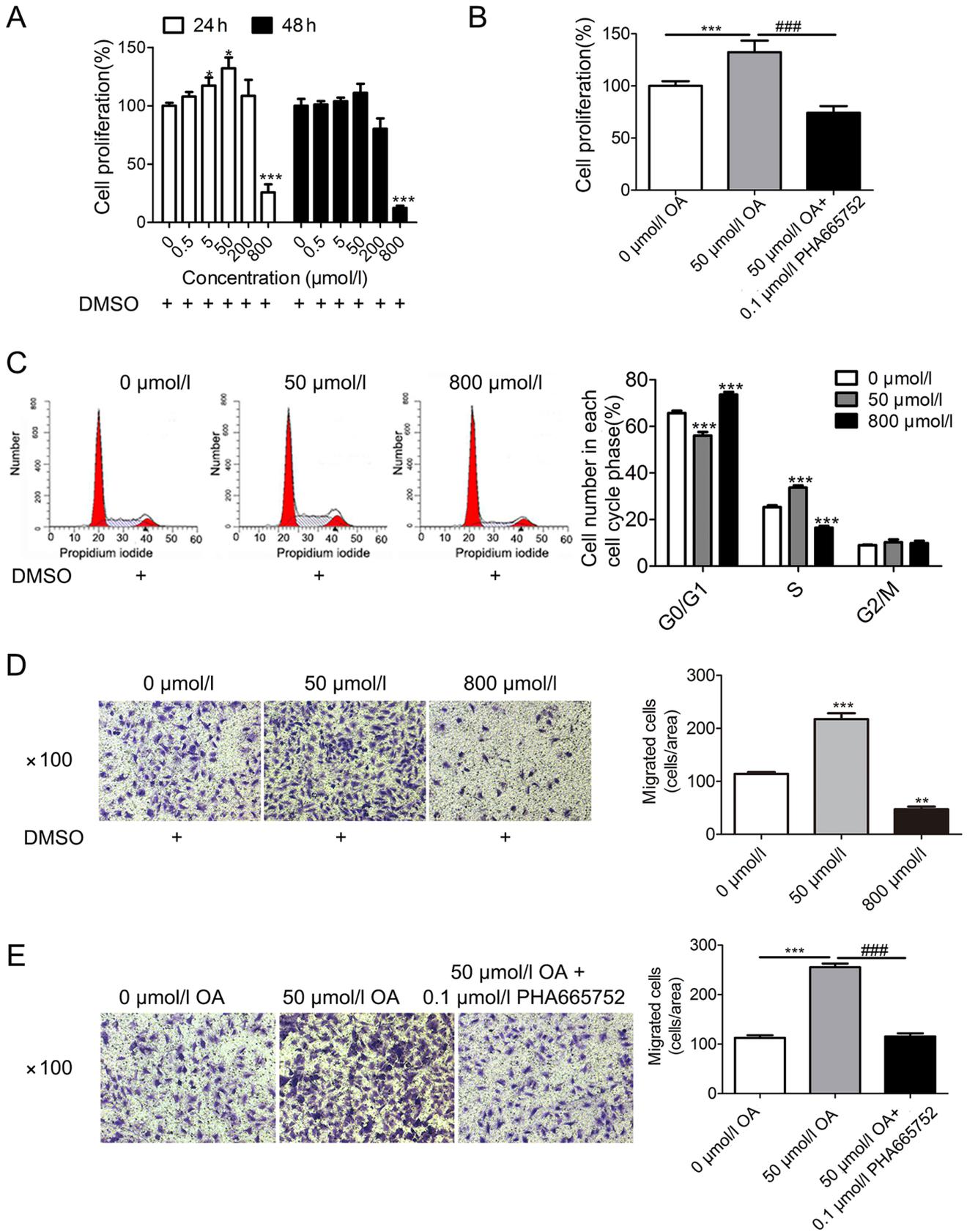


Figure 1. Effect of OA on the proliferation, migration and cell cycle of A7r5 cells. (A) Cell Counting Kit-8 assays showed that OA significantly promoted (at 5 and 50 μmol/l) or inhibited (at 800 μmol/l) A7r5 cell proliferation in a time- and concentration-dependent manner at 24 and 48 h. (B) Optimal effect of 50 μmol/l OA for 24 h on cell proliferation was mitigated by 0.1 μmol/l PHA665752, which is a selective inhibitor of HGF. (C) Flow cytometry assays at 24 h identified that low concentrations of OA (50 μmol/l) reduced the number of cells in the G₀/G₁ phase and increased the number of cells in the S phase. However, high concentrations of OA (800 μmol/l) increased the number of cells in G₀/G₁ phase and decreased the number of cells in S phase. (D) Transwell assays at 24 h demonstrated that OA at low concentrations (50 μmol/l) promoted cell migration, but a high concentration (800 μmol/l) suppressed migration. (E) OA (50 μmol/l) promoted cell migration at 24 h, which was mitigated by PHA665752 (0.1 μmol/l). Data are presented as the mean ± SD (n=3). Magnification, x100. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μM OA group; ###P<0.001 vs. 50 μmol/l OA group. OA, oleic acid.

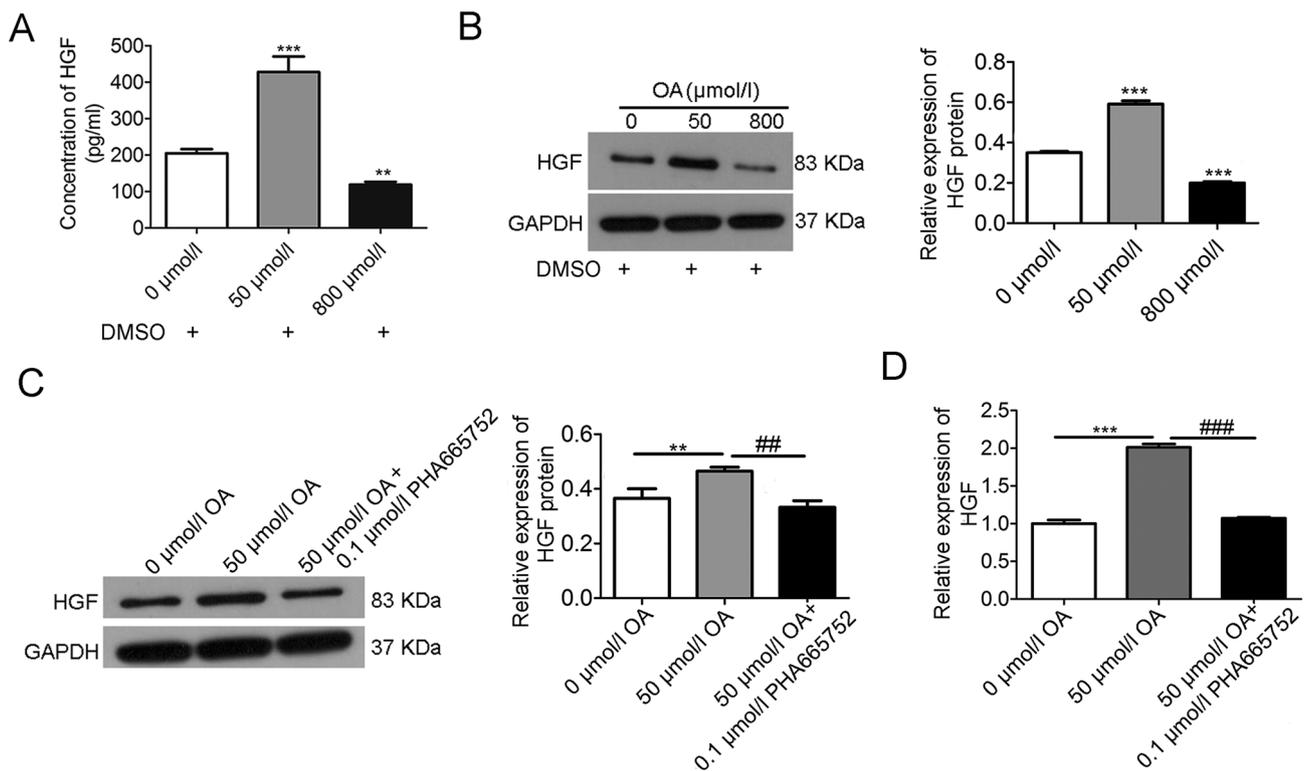


Figure 2. Effects of OA on the expression of HGF in A7r5 cells. (A) ELISA results at 24 h identified that low concentrations of OA (50 μmol/l) upregulated the level of HGF, but HGF was downregulated at high concentrations (800 μmol/l) of OA. (B) Western blot analysis at 24 h demonstrated that low concentrations of OA (50 μmol/l) increased the expression of HGF, but high concentrations of OA (800 μmol/l) downregulated this expression. (C) Western blot analysis showed that the increased HGF expression at low concentrations of OA (50 μmol/l) was mitigated by PHA665752 (0.1 μmol/l). (D) Reverse transcription-quantitative PCR analysis at 24 h indicated that the increased expression of HGF at low concentrations of OA (50 μmol/l) was mitigated by PHA665752 (0.1 μmol/l). Data are presented as the mean ± SD (n=3). **P<0.01 and ***P<0.001 vs. 0 μM OA group; #P<0.01 and ###P<0.001 vs. 50 μmol/l OA group. OA, oleic acid; HGF, hepatocyte growth factor.

(50 μmol/l OA) but downregulated it at high concentrations (800 μmol/l OA), as determined by ELISA (Fig. 2A) and western blotting (Fig. 2B). Moreover, the increased expression of HGF at low concentrations of OA (50 μmol/l) was mitigated by 0.1 μmol/l PHA665752, as observed via western blotting (Fig. 2C) and RT-qPCR (Fig. 2D).

Effects of HGF inhibition and p38 MAPK inhibition on OA-induced A7r5 cell proliferation and migration. To investigate the mechanism of OA-induced A7r5 cell proliferation and migration via HGF signaling, the p38 inhibitor SB203580 was used, which inhibits PKB phosphorylation. The results demonstrated that the A7r5 cell proliferation induced by OA (50 μmol/l) was mitigated by PHA665752 (0.1 μmol/l) or SB203580 (2 μmol/l) (Fig. 3A). Moreover, A7r5 cell migration was mitigated by SB203580 (2 μmol/l) (Fig. 3B). The effects of OA on p38 and p-p38 expression levels were then determined via western blotting (Fig. 3C), and the data revealed that OA treatment increased p-p38 expression at low concentrations (50 μmol/l OA) but downregulated it at high concentrations (800 μmol/l OA). Moreover, the increased expression of p-p38 at low concentrations of OA (50 μmol/l) was mitigated by PHA665752 (0.1 μmol/l) (Fig. 3D) or SB203580 (2 μmol/l) (Fig. 3E). By contrast, there was no notable difference in the expression of p38.

In addition, via preliminary experiments, it was observed that 1 mmol/l DMSO, 2 μmol/l SB203580 or 0.1 μmol/l

PHA665752 did not affect the proliferation and migration of A7r5 cells (data not shown), which was consistent with previous studies (29-34).

Discussion

The present study demonstrated that OA could promote A7r5 cell proliferation at a low concentration and over a short time length, but OA inhibited A7r5 cell proliferation at a high concentration and over longer times, as demonstrated using CCK-8 assays, cell cycle analysis and Transwell assays. Interestingly, the A7r5 cell proliferation and migration induced by OA (50 μmol/l) were mitigated by PHA665752 (0.1 μmol/l), a selective inhibitor of HGF. Using ELISA and western blotting, it was observed that HGF expression was increased in A7r5 cells at low doses of OA (50 μmol/l) but was decreased using high doses of OA (800 μmol/l). However, the increased expression of HGF induced by OA (50 μmol/l) was also mitigated by the HGF inhibitor PHA665752 (0.1 μmol/l).

HGF, an angiogenic factor, is associated with the risk of coronary heart disease, stroke and atherosclerosis (36,37). Circulating HGF has been proposed as a potential clinical biomarker for CVD (38). Previous studies have shown that high concentrations of HGF were significantly associated with the progression of atherosclerosis (39). HGF and its receptor c-MET serve an important role in endothelial injury and repair, angiogenesis, cell migration, cell survival and

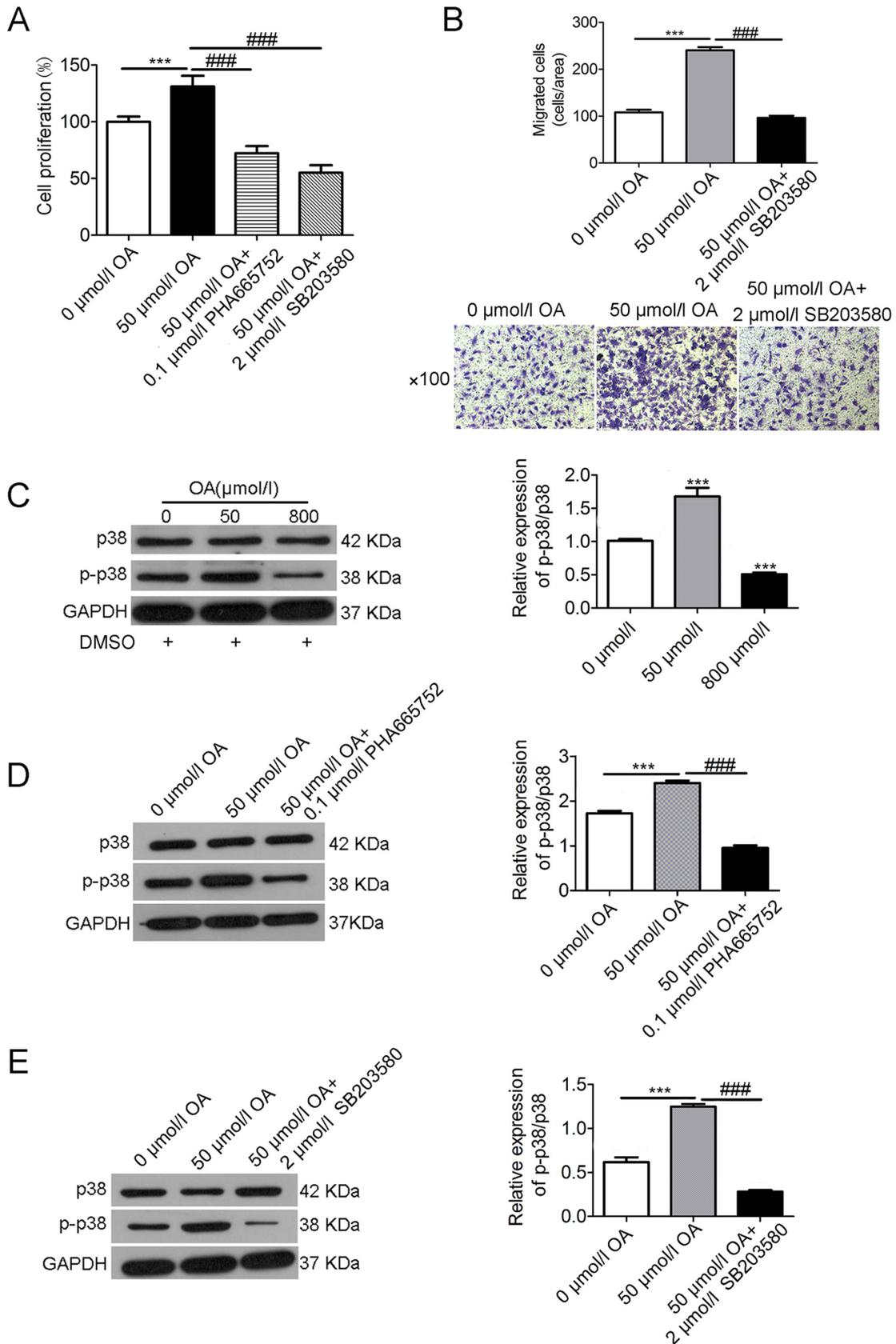


Figure 3. Effects of HGF inhibitors and p38 MAPK inhibitors on OA induced A7r5 cell proliferation and migration. (A) Cell Counting Kit-8 assays at 24 h indicated that the A7r5 cell proliferation induced by OA (50 $\mu\text{mol/l}$) was significantly suppressed by PHA665752 (0.1 $\mu\text{mol/l}$) or SB203580 (2 $\mu\text{mol/l}$). (B) Cell migration with 50 $\mu\text{mol/l}$ OA treatment for 24 h was mitigated by 2 $\mu\text{mol/l}$ SB203580, a selective inhibitor of p38 MAPK. Magnification, $\times 100$. (C) Western blotting at 24 h showed that OA treatment increased p-p38 expression at low concentrations (50 $\mu\text{mol/l}$), but p-p38 expression was downregulated at high concentrations (800 $\mu\text{mol/l}$). (D) Western blotting at 24 h revealed that increased expression of p-p38 at low concentrations of OA (50 $\mu\text{mol/l}$) was mitigated by PHA665752 (0.1 $\mu\text{mol/l}$). (E) Western blot analysis at 24 h demonstrated that increased expression of p-p38 at low concentrations of OA (50 $\mu\text{mol/l}$) was mitigated by SB203580 (2 $\mu\text{mol/l}$). Data are presented as the mean \pm SD (n=3). ***P<0.001 vs. 0 μM OA group; ###P<0.001 vs. 50 $\mu\text{mol/l}$ OA group. OA, oleic acid; p-, phosphorylated.

anti-inflammatory responses in multiple cell types (16,40). The present study used the HGF inhibitor PHA665752 (27,41,42) to examine whether there was an association between the effect of OA and the HGF signaling pathway. The current results suggested that the effect of OA on proliferation and migration in A7r5 cells was mitigated by PHA665752. Moreover, the mRNA and protein expression levels of HGF induced by OA were suppressed by PHA665752 in A7r5 cells. These results indicated that OA promoted cell proliferation and migration via HGF signal transduction, which is helpful for further understanding the effects and mechanisms driving OA's effect on CVD (38).

p38 MAPK signaling has been reported to serve a role in VSMC proliferation (23) and HGF secretion from human astrocytoma cells (25). The present study used the p38 MAPK inhibitor SB203580 to evaluate whether there was an association between the effects of OA and p38 MAPK signaling, and p38 MAPK served as the positive control for several of the assays. The current data demonstrated that A7r5 cell proliferation and migration induced by OA (50 $\mu\text{mol/l}$) were suppressed by SB203580 (2 $\mu\text{mol/l}$), and the increased expression level of p-p38 at low concentrations of OA (50 $\mu\text{mol/l}$) was also mitigated by PHA665752 (0.1 $\mu\text{mol/l}$) or SB203580 (2 $\mu\text{mol/l}$). However, there was no notable difference in the expression level of p38. The present findings suggested that the effect of OA on cell proliferation and migration was mitigated by SB203580 in A7r5 cells, and the expression of p-p38 protein induced by OA was also suppressed by SB203580 in A7r5 cells. Furthermore, the results indicated that OA promoted cell proliferation and migration by regulating p38 phosphorylation, and that p38 MAPK signal transduction may serve an important role in the VSMC proliferation and migration induced by OA. Collectively, these data suggested that the OA-induced stimulation of the proliferation and migration of VSMCs may be associated with increased expression levels of HGF and p-p38. Therefore, the results of other studies (19,23) and the present study support the hypothesis that p38 MAPK, as a downstream mediator, regulates the activity of HGF induced by OA in A7r5 cells.

The aforementioned results support the following two principal findings. First, low (physiological) concentrations of OA induce VSMC proliferation and migration, which is consistent with previous studies (43,44). Second, the proliferation and migration of VSMCs induced by OA appear to be mediated by HGF and the p38 MAPK pathway. To the best of our knowledge, the current study demonstrated for the first time that OA stimulated the proliferation and migration of VSMCs via the HGF and p38 MAPK pathways *in vitro*.

Several signaling pathways, including the PI3K/Akt (28), reactive oxygen species (ROS) (45), AMP-activated protein kinase (AMPK)/endothelial nitric oxide synthase/FAS (46) and AMPK pathways (47), have been reported to serve a role in the proliferation and migration of VSMCs. The present findings suggest that HGF and p38 MAPK represent a new signaling pathway that participates in the proliferation and migration of VSMCs stimulated by OA.

The present study also suggested that the effect of OA on the proliferation and migration of A7r5 cells could be

regulated by the HGF and p38 MAPK signaling pathways, and the proliferation and migration of VSMCs induced by OA were associated with increased expression levels of HGF and p-p38.

Several studies have revealed that OA treatment can induce apoptosis in neuronal cells (48), carcinoma cells (49) and VSMCs (50,51), and is respectively associated with dephosphorylation of Bad, increasing ROS production, or caspase 3 activity, autophagy and LOX-1 upregulation. These results may explain the toxicity or inhibition of treatment with OA at high concentrations and over longer time frames on the proliferation and migration of A7r5 cells, which was observed in the current experiments. There are five limitations to the present study. First, the mechanisms involved in the up- or downregulation of the expression levels of HGF and p-p38 in A7r5 cells induced by OA need to be studied in the future. Second, the effects on cell cycle markers using small interfering RNAs or short hairpin RNAs, and other related signaling molecules, need to be evaluated further. Third, the present data indicated that OA had an effect on both the proliferation and migration of A7r5 cells, but the results of migration experiments may have been affected by differentiated proliferation (52). Fourth, the expression levels of contractile and synthetic markers in VSMCs should be evaluated. Fifth, there was the absence of PHA665752 or SB203580 alone control groups in this study.

In conclusion, the effect of OA on A7r5 cell proliferation and migration was both time- and concentration-dependent, and low concentrations and short time frames promoted cell proliferation and migration, while high concentrations and long time frames inhibited cell proliferation and migration. Treatment with OA at 24 h could promote A7r5 cell proliferation and migration, but the effect of OA in promoting cell proliferation and migration disappeared at 48 h, which was consistent with, but not precisely replicated, in our prior experiments (12,13). These findings suggested that the promoting or inhibiting effects of glyceryl trioleate and medium chain TG on the proliferation of A7r5 cells (low concentrations and short time periods promoting cell proliferation; high concentrations and long time periods inhibiting cell proliferation) were not associated with cell apoptosis. Our previous findings (12,13) are indirectly supported by a study from Belal *et al* (53), which revealed that OA can promote the proliferation of bovine satellite cells without apoptosis or necrosis. Oxidative stress and ROS production may explain the current experiment results obtained from treatment with OA for 48 h or at high concentrations (49), and long treatment durations or high concentrations of OA treatment may activate the MAPK pathway by oxidative stress to inhibit cell proliferation (19,20). Collectively, it was indicated OA, HGF and p38 MAPK may be potential therapeutic targets for CVDs (such as atherosclerosis) (37). The present results provide novel insights into the biological effects of OA, HGF and p38 MAPK, and new evidence for the effect and mechanism of OA regulation on the proliferation and migration of VSMCs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MY conceived and designed the study. JL performed the experiments and acquired the data. MY and JL confirm the authenticity of all the raw data. MY, JL and TC analyzed the data. MY, JL and TC drafted of the manuscript. MY and TC contributed to the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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