Hydrogen peroxide promotes the expression of angiopoietin like 4 in RAW264.7 macrophages via MAPK pathways

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Abstract. Previous studies including some vivo experiments and large scale clinical trials have indicated that angiopoietin like 4 (ANGPTL4) is involved in atherosclerosis. However, the specific mechanism underlying the process remains unresolved. Similarly, cumulative evidence indicated that hydrogen peroxide (H_2O_2) is closely related to the occurrence and development of atherosclerosis. The current study investigated whether H_2O_2 treatment can affect ANGPTL4 release in macrophage cells cell viability assay, western blot analysis, ELISA and immunofluorescence. It was determined that treatment with 0.25 and 0.5 mM H_2O_2 resulted in a significant increase in ANGPTL4 protein expression in macrophage cells. Mitogen-activated protein kinase (MAPK) pathways were implicated in the secretion of ANGPTL4 regulated by H_2O_2, and specific inhibitors of MAPK1 (also known as ERK) and p38 MAPK significantly decreased H_2O_2 induced ANGPTL4 protein expression. Accordingly, it was demonstrated that ANGPTL4 expression was regulated by H_2O_2 via ERK and p38 MAPK, but not the MAPK8 (also known as JNK) pathway. In view of the effects of H_2O_2 and ANGPTL4 on atherosclerosis, the influence of H_2O_2 on ANGPTL4 provided new insight into the mechanism of atherosclerosis.

Introduction

Angiopoietin like 4 (ANGPTL4) was first identified as a novel protein with a similar structure to other angiopoietin-like proteins. Angiopoietin-like proteins have a structure composed of an N-terminal coil-coil structure and a C-terminal fibrinogen-like domain (1). ANGPTL4 is primarily expressed in the liver, adipose tissues, heart, skeletal muscle, intestine, blood plasma, ovaries and the placenta in humans (2). ANGPTL4 is involved in many physiological and pathological conditions including lipid metabolism, glucose homoeostasis, inflammation, kidney disease, wound healing, cell differentiation, tumorigenesis, angiogenesis, vascular permeability and redox regulation (3). Peroxisome proliferator-activated receptors (PPARs) transcriptionally stimulate ANGPTL4 expression via the PPAR-response element and transforming growth factor β (TGF-β), which transcriptionally stimulates ANGPTL4 expression via the TGF-β responsive enhancer. Furthermore, PPARs and TGF-β can synergistically or antagonistically regulate ANGPTL4 expression (4). Hypoxia also elevates the expression of ANGPTL4 by transcriptional regulation by hypoxia-inducible factor 1 (HIF-1) (5). PPARs and HIF-1 have a synergistic interaction in regulating ANGPTL4 transcription by changing the conformational proximity of corresponding response elements (6).

Reactive oxygen species (ROS) are a class of chemically reactive oxygen-containing compounds, including superoxide, hydroxyl radicals and hydrogen peroxide (H_2O_2), which serve important roles in normal biochemical functions and abnormal pathological processes (7). The accumulation of ROS can cause protein dysfunction and DNA damage. ROS also function as chemical messengers to activate signaling pathways that are involved in cell proliferation, differentiation, and apoptosis (8). Of particular interest is H_2O_2, which is mainly produced in biological systems by the dismutation of the superoxide anion in a reaction carried out by the enzyme superoxide dismutase. H_2O_2 is not only thought to function as a cellular damaging agent that reacts toward lipids, proteins, and DNA, but also acts as a crucial mediator of intracellular signaling. As a crucial second messenger, H_2O_2 can activate a myriad of signaling pathways of which the best known are the mitogen-activated protein kinase (MAPK) pathways (9). The MAPKs are highly conserved serine/threonine protein kinases that function in various fundamental cellular processes, including proliferation, differentiation, motility, apoptosis and survival (10). Increasing the concentration of H_2O_2 promotes phosphorylation of apoptosis signal-regulating kinase 1 (ASK1). ASK1 is a MAPK that activates MAPK8 (commonly known as JNK) and p38 MAPK, but not MAPK1 (commonly known as ERK) (11). Besides, H_2O_2 may activate MAPKs

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pathways via other mechanisms such as the nuclear factor-κB MAPK pathway (12,13). Furthermore, a previous study demonstrated that increased p38 activity produced a positive feedback to enhance ROS generation by upregulating nicotinamide adenine dinucleotide phosphate-oxidase, H_2O_2 and p38 to develop a positive feedback loop (14). Accordingly, MAPKs act on respiratory burst oxidase homologs, a type of NADPH oxidase in plants, and thus accelerate ROS production (15).

ERK and JNK are both involved in the induction of ANGPTL4 by para-methoxyamphetamine (PMA) in human airway smooth muscle cells, and a role for p38 in PMA-induced ANGPTL4 increase has been excluded (16). In addition, the ERK inhibitor, U0126, and the JNK inhibitor, SP600125, greatly inhibit the increase in ANGPTL4 expression (16). In the human glioblastoma cell line LN229, ANGPTL4 expression is significantly induced by epidermal growth factor receptor variant type III (EGFRIII). In this process, the MAPK pathway, especially ERK is activated with the transcription factor c-Myc, which regulates ANGPTL4 transcription (17).

Considering the importance of MAPK signaling pathways with regard to H_2O_2 and ANGPTL4, it was hypothesized that there may be a mutual effect of H_2O_2 on ANGPTL4. To the best of our knowledge, the potential role of H_2O_2 in the regulation of ANGPTL4 release has not been investigated previously. The present study was therefore undertaken to determine the effects of H_2O_2 treatment on ANGPTL4 release in macrophage cells.

Materials and methods

Materials. H_2O_2 (cat. no. 323381) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and diluted in PBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS; cat. no. F8240) was purchased from Hangzhou Four Seasons Green Engineering Materials Co., Ltd., (Hangzhou, China) and Dulbecco's modified Eagle's medium (DMEM; cat. no. C11995500BT) from Gibco; Thermo Fisher Scientific, Inc. Primary antibody against α-tubulin (cat. no. 60031-1-ig) was purchased from Wuhan SanYing Biotechnology, Inc. (Wuhan, China). Primary antibody against ANGPTL4 (cat. no. 40-9800) was obtained from Invitrogen; Thermo Fisher Scientific, Inc. Primary antibodies against phosphorylated (p-)p38 MAPK (cat. no. 4511), p38 MAPK (cat. no. 8690), p-ERK1/2 (cat. no. 4370), ERK1/2 (cat. no. 4695), p-JNK (cat. no. 4668) and JNK (cat. no. 9252) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), as were U0126 (cat. no. 9903) and SB203580 (cat. no. 5633). SP600125 (cat. no. S5567) was purchased from Sigma-Aldrich; Merck KGaA. Goat anti-rabbit secondary antibody (cat. no. 2301) and rabbit anti-mouse secondary antibody (cat. no. ZB2305) were obtained from OriGene Technologies, Inc. (Beijing, China). The primary anti-ANGPTL4 antibody (cat. no. 251458) used for cell immunofluorescence was obtained from Abbiotec LLC (San Diego, CA, USA) and the goat anti rabbit fluorescent secondary antibody (cat. no. A-11034) was from Applied Biosystems; Thermo Fisher Scientific, Inc. Cell Counting Kit-8 (CCK-8) was obtained from BestBio (Shanghai, China) and the mouse ANGPTL4 ELISA kit (cat. no. KB18884) was purchased from Jiang Lai Bio-Technology (Shanghai, China).

Cell culture and treatment. Murine macrophage RAW264.7 cells were obtained from Shanghai Type Culture Collection (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin, and incubated at 37˚C in 5% CO_2. For the experiments, the cells were stimulated for 24 h with H_2O_2 at various concentrations. The specific inhibitors U0126, SB203580 and SP600125 were dissolved in DMSO to appropriate concentrations and used at 20, 40 and 10 mM, respectively. For pretreatments, cells were incubated for 30 min with SP600125, 90 min with SB203580, or 90 min with U0126, prior to H_2O_2 treatment.

Cell viability assay. Cell viability was evaluated by CCK-8 assay. The cells were cultured at a density of 10^5 cells/well on 96-well plates in 100 μl culture medium as described above. Following 24 h of culturing, the cells were stimulated with or without H_2O_2 (0.25 or 0.5 mM) for 24 h. Then the cells of each microwell were incubated with 10 μl CCK8 and 90 μl culture medium for 2 h at 37˚C and then the absorption values were measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was expressed as a percentage of the control.

Western blot analysis. Following pretreatment and H_2O_2 treatment, cells and culture media were separated by centrifugation at 4˚C for 20 min at 2,000 x g. Total protein was extracted from cells for western blotting, while the culture medium was saved for ELISAs. Total protein was extracted using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Beijing, China), which contained 0.1 M phenylmethylsulfonyl fluoride. Following washing with PBS, grinding, lysis and centrifugation (at 4˚C, 13,000 x g, 15 min), the supernatant was collected. Then proteins (100 μg per lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked by incubation for 90 min at room temperature in 5% non-fat milk in TBS + 0.2% Tween-20 (TBST). Membranes were incubated at 4˚C overnight with the following specific primary antibodies: α-tubulin (1:5,000), ANGPTL4 (1:1,000), p-p38 MAPK (1:1,000), p38 MAPK (1:1,000), p-ERK1/2 (1:1,000), ERK (1:1,000), p-JNK (1:1,000) and JNK (1:1,000). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000) in 5% non-fat milk in TBST for 1 h at room temperature. Finally, bands were detected using chemiluminescence kit (EMD Millipore Billerica, MA, USA) and quantified with the FluorChem E system (ProteinSimple; Bio-Techne, Minneapolis, MN, USA).

Immunofluorescence. Cells (~1x10^5/ml) were cultured on cover slips and then treated with or without H_2O_2 for 24 h. Cells were then fixed in 4% paraformaldehyde for 15 min prior to extraction with 0.5% Triton X-100. Then, nonspecific antibodies were blocked by incubation with goat serum (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 30 min at 37˚C and then incubated with anti-ANGPTL4 antibody at 4˚C overnight, followed by incubation with AlexaFluor-488 goat anti-rabbit IgG (cat. no. A-11034; Thermo Fisher Scientific, Inc.) for 45 min at 37˚C. Slides were then incubated with 4',6-diamidino-2-phenylindole to counterstain and fluorescent signals were

detected using a light microscope; >3 fields of view were examined in ~9 slides.

ELISA. The culture medium in 6-well plates was collected as described above, and the quantity of ANGPTL4 protein in the culture medium was determined by ELISA. The absorbance at 450 nm was detected using a microplate reader and ANGPTL4 levels were calculated according to a standard curve.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) and all experimental data are presented as the mean ± standard deviation of at least three independent experiments. Comparisons between two groups were performed using two-tailed Student's t-tests. Multiple comparisons were performed by one way analysis of variance followed by Bonferroni's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H_2O_2 induces ANGPTL4 release in RAW264.7 macrophage cells. To study the effect of H_2O_2 on ANGPTL4 protein expression, 0, 0.125, 0.25, 0.375 and 0.5 mM H_2O_2 was added to macrophage cells. The lowest concentration, 0.125 mM, increased ANGPTL4 protein levels slightly compared with control, but was not statistically significant (data not shown). As demonstrated by immunofluorescence, incubation of cells with 0.25 and 0.5 mM H_2O_2 for 24 h resulted in significantly more ANGPTL4 in the cytoplasm and the nucleus compared with control ($P<0.001$; Fig. 1A). Significant upregulation of ANGPTL4 protein expression compared with the control was also observed by western blotting (Fig. 1B). In addition, significantly more ANGPTL4 was secreted into the medium in cells treated with 0.25 and 0.5 mM H_2O_2 compared with control, as detected by ELISA ($P<0.05$; Fig. 1C).

Regulation of ANGPTL4 by H_2O_2 is mediated by MAPK pathways. To gain insight into the underlying mechanism of H_2O_2-induced ANGPTL4 release, the roles of MAPK signaling pathways were investigated. The level of ERK1/2 (Fig. 2A), p38 MAPK (Fig. 2B) and JNK (Fig. 2C) phosphorylation in cells stimulated with 0.25 and 0.5 mM H_2O_2 was significantly increased compared with control. It was therefore concluded that H_2O_2 effectively induced phosphorylation of ERK1/2, p38 MAPK and JNK proteins in the macrophage cells; however, this conclusion is insufficient to determine that H_2O_2-induced ANGPTL4 expression is dependent on MAPK pathway activation.

U0126 and SB203580 inhibit H_2O_2-induced ANGPTL4 release in RAW264.7 macrophage cells. To explore this issue further, the effects of specific inhibitors of p38 MAPK (SB203580), JNK (SP600125) and ERK1/2 (U0126) on H_2O_2-induced ANGPTL4 expression were investigated. As illustrated in Fig. 3A, compared with the uninhibited samples, preincubation of the cells with 20 mM UO126 for 90 min to block ERK1/2 activation significantly inhibited overexpression of ANGPTL4 protein induced by H_2O_2 ($P<0.01$). Similarly, preincubation of cells with 40 mM SB203580 for 90 min to block p38 MAPK pathway activation inhibited overexpression of ANGPTL4 protein induced by H_2O_2 compared with uninhibited samples

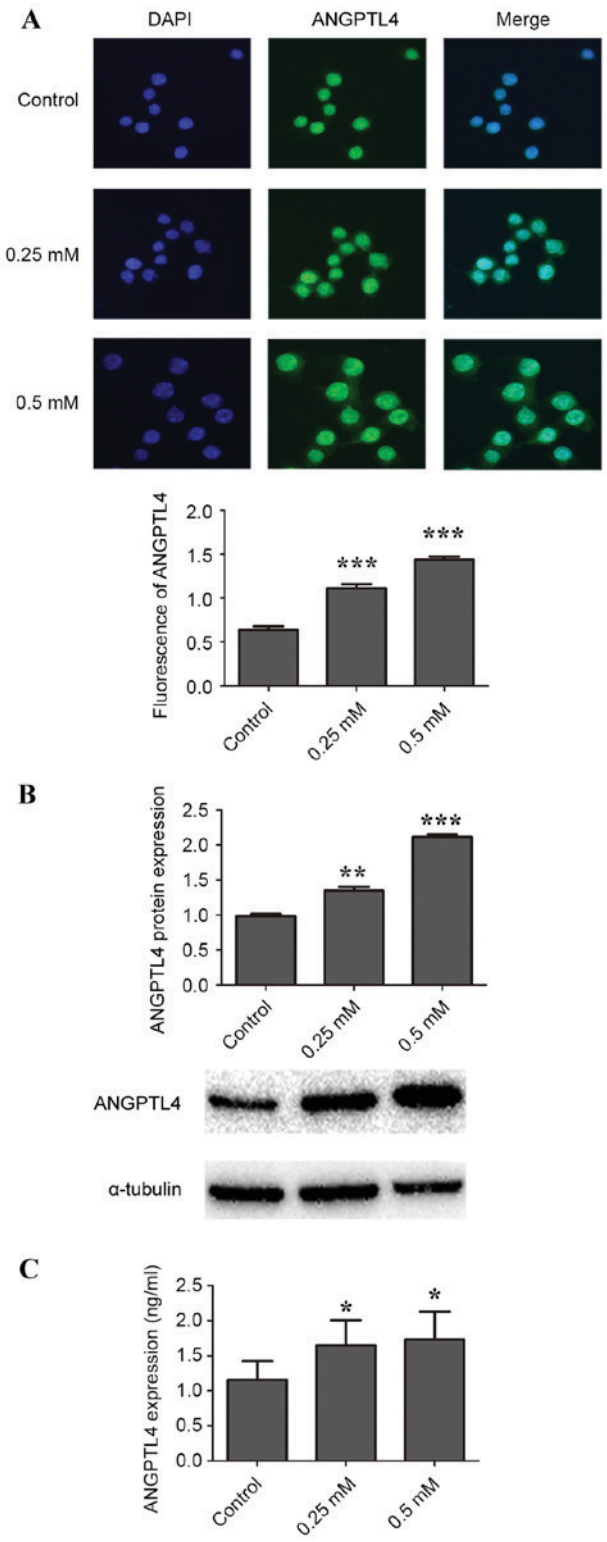

Figure 1. Effect of H_2O_2 on ANGPTL4 protein expression in RAW264.7 macrophage cells. (A) Cells were stimulated with 0 (control), 0.25 or 0.5 mM H_2O_2 for 24 h, then ANGPTL4 protein expression in the cytoplasm and nucleus was determined by immunofluorescence. Cells were stimulated with 0 (control), 0.25 or 0.5 mM H_2O_2 for 24 h, then ANGPTL4 protein expression in (B) cell lysates was determined by western blotting, with α-tubulin as a loading control, and (C) ANGPTL4 protein expression in the culture medium was examined by ELISA. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. control. ANGPTL4, angiopoietin like 4.

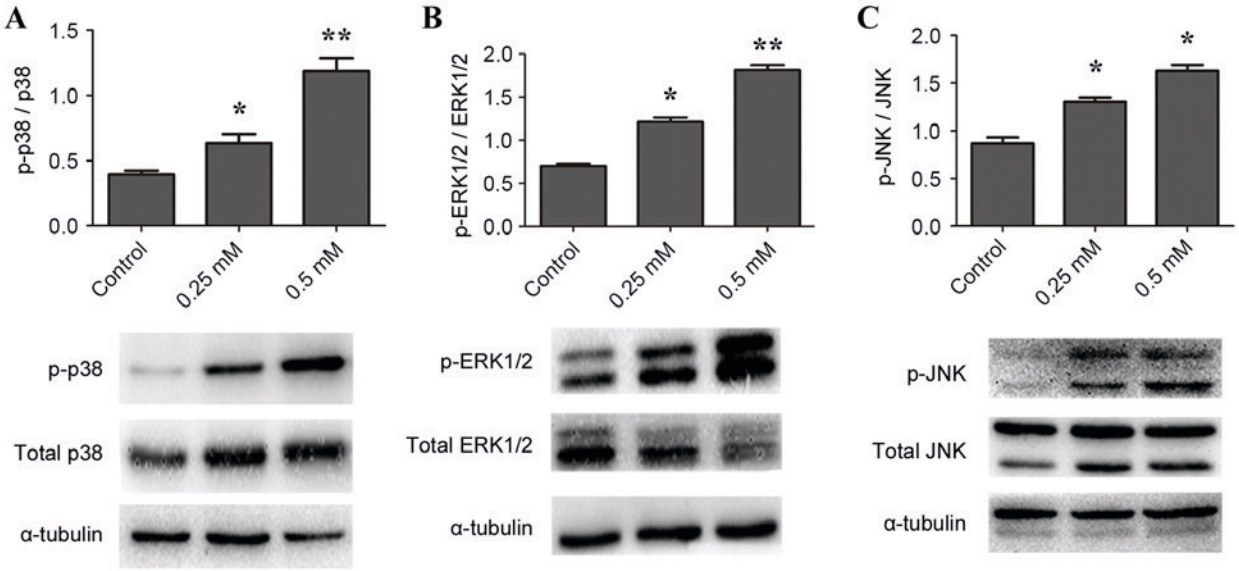

Figure 2. H_2O_2 activates ERK1/2, p38 MAPK and JNK protein phosphorylation expression in RAW264.7 macrophages. Cells were stimulated with 0 (control), 0.25 or 0.5 mM H_2O_2 for 24 h, then protein expression in cell lysates was measured by western blotting. Cell lysates were probed for (A) p- and total ERK1/2, (B) p- and total p38 MAPK and (C) p- and total JNK, with α-tubulin as loading control. *P<0.05, **P<0.01 and ***P<0.001 vs. control. ERK1/2, MAPK1; MAPK, mitogen-activated protein kinase; JNK, MAPK8; p-, phosphorylated.

(P<0.05; Fig. 3B). However, compared with uninhibited samples, H_2O_2-induced ANGPTL4 protein levels were not affected in cells preincubated with 10 mM SP600125 for 30 min to block JNK pathway activation (Fig. 3C). Due to the toxicity to the macrophage cells, it was not possible to increase the concentration of SP600125 further. Together these results indicated that inhibition of p38 MAPK and ERK1/2, but not JNK, attenuated H_2O_2-induced ANGPTL4 release in macrophage cells.

Discussion

In the present study, ANGPTL4 was identified as a protein that was upregulated in Raw264.7 macrophages exposed to high concentrations of exogenous H_2O_2. Macrophages are immune cells that take part in immune response associated disease, including atherosclerosis. Atherosclerosis primarily refers to a maladaptive immune response caused by the accumulation of cholesterol-laden macrophages in the artery wall (18,19). Macrophages serve a key role via transition to foam cells that trigger the formation of an atherosclerotic lesion (20,21). Furthermore, macrophage cell autophagy participates in atherosclerosis by exerting a protective influence (22). Macrophage cells secrete various kinds of pro-inflammatory cytokines, which form an indispensable part of the inflammation reaction in the genesis of atherosclerosis (23,24)., ANGPTL4 is highly expressed in macrophages. A previous study has indicated that ANGPTL4 decreases the uptake of oxidized low-density lipoprotein (ox-LDL) in macrophage cells and consequently suppresses foam cell formation which prevents the pathological development of atherosclerosis (25). By contrast, H_2O_2 has been demonstrated to promote the oxidative modification of LDL into ox-LDL and accelerate foam cell formation (26). Therefore, ANGPTL4 and H_2O_2 take opposite roles in the formation of foam cells. The present study linked ANGPTL4 and H_2O_2 via MAPK pathways. p38, ERK and JNK are all activated by high concentrations of H_2O_2 in macrophages. However, only the ERK inhibitor U0126 and the p38 inhibitor SB203580 were demonstrated to suppress ANGPTL4 expression induced by H_2O_2. Although the JNK pathway was activated, the special inhibitor SP600125 did not significantly decrease H_2O_2-induced ANGPTL4 expression. In different cell types, H_2O_2 stimulates different subgroups of MAPKs and the mechanism is not well understood (27). ANGPTL4 interacts with integrins to stimulate NADPH oxidase-dependent production of ROS, particularly O_2. A high ratio of O_2:H_2O_2 activates the ERK pathways (28). Interestingly, the C-terminal of ANGPTL4 can inhibit the phosphorylation of ERK induced by b-FGF in turn (29).

ANGPTL4 is a lipid metabolism associated factor and abundant evidence has demonstrated that the hypertriglyceridemic effect of ANGPTL4 is mainly attributable to inhibition of lipoprotein lipase (LPL), the enzyme that hydrolyses triglycerides (30,31). To date, the role of ANGPTL4 in atherosclerosis remains controversial. For the past few decades, the emphasis of research of ANGPTL4 on atherosclerosis has been concentrated on the regulation of lipid metabolism and it is still unknown whether ANGPTL4 directly affects the genesis and development of atherosclerosis. Some studies suggest that ANGPTL4 serves a protective role in atherosclerosis, and the mechanism is independent of the influence of ANGPTL4 on triglyceride levels. Indeed, one study demonstrated that gene knockout of ANGPTL4 is sufficient to protect against the development and progression of atherosclerosis by suppressing formation of foam cells and thereby reducing the atherosclerotic lesion size (32). In consideration of the diverse physiological functions of ANGPTL4, intensive experiments are required to resolve these discrepancies.

Besides promoting foam cell formation, H_2O_2 has critical roles in the pathogenesis of atherosclerosis by regulating the migration of smooth muscle cells, monocyte infiltration and apoptosis of vascular cells during advanced atherosclerotic

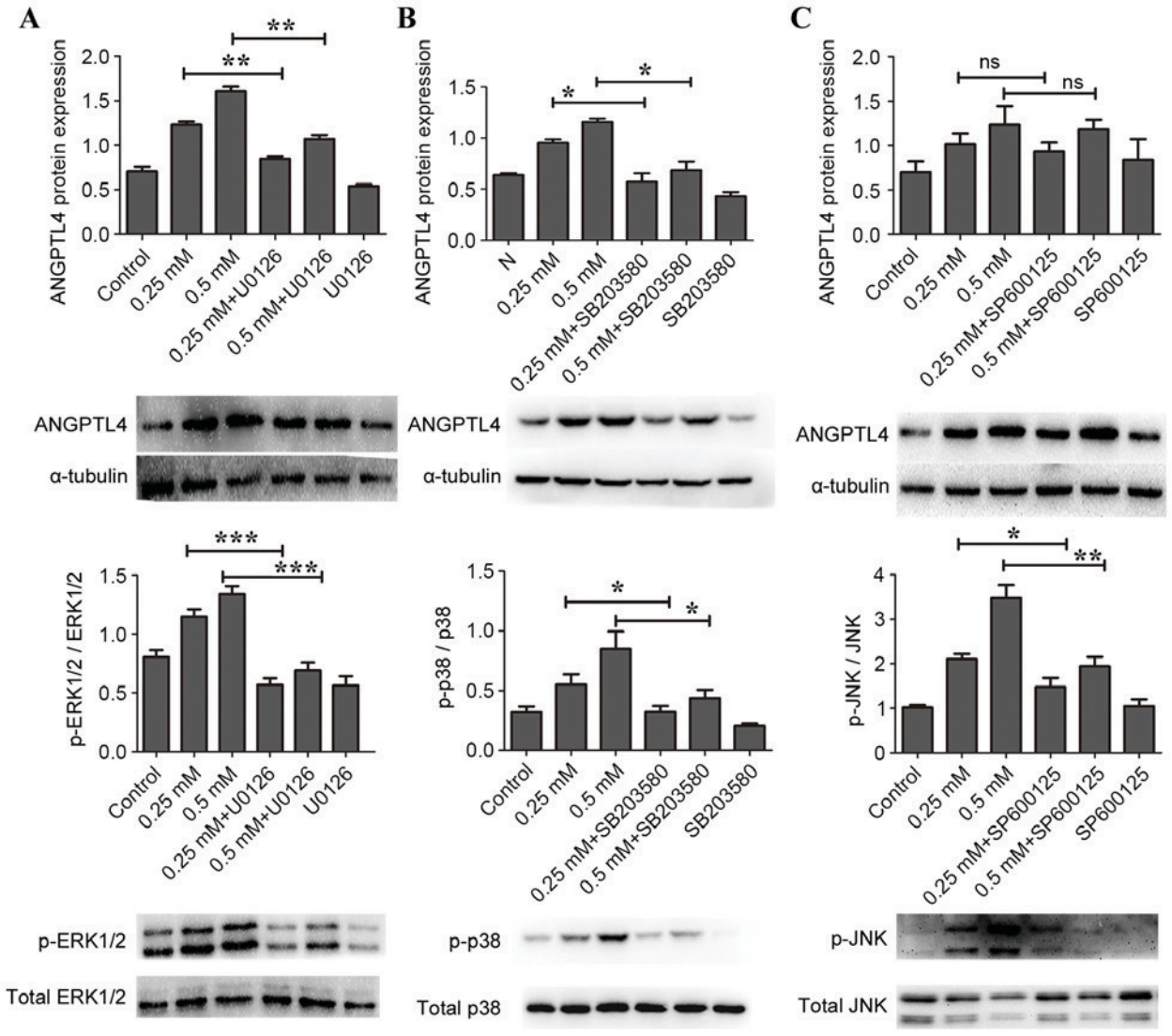

Figure 3. Effects of MAPK inhibitors on H_2O_2-induced ANGPTL4 release in RAW264.7 macrophage cells. Cells were pretreated with specific inhibitors for ERK1/2 (20 mM U0126), p38 MAPK (40 mM SB203580) or JNK (10 mM SP600125), followed by stimulation with 0, 0.25 or 0.5 mM H_2O_2 for 24 h. Protein expression levels were then determined by western blotting, with α-tubulin as loading control. (A) ANGPTL4, p-ERK1/2 and total ERK1/2. (B) ANGPTL4, p-p38 MAPK and total p38 MAPK. (C) ANGPTL4, p-JNK and total JNK. *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets. MAPK, mitogen-activated protein kinase; ANGPTL4, angiopoietin like 4; ERK1/2, MAPK1; JNK, MAPK8; p-, phosphorylated; Control, 0 mM H_2O_2 and no inhibitor; ns, not significant.

process (33-35). H_2O_2 has also been reported as inducer of autophagy which is involved in lipid homeostasis and dyslipidemias associated with atherosclerosis. During the development of atherosclerosis, H_2O_2 may inhibit smooth muscle cell migration by mediating the downregulation of myosin phosphatase target subunit 1 (36,37). Numerous studies have revealed that oxidative stress caused by H_2O_2 is a major factor contributing to the damage of endothelial cells; the resulting endothelial injury is an important pathological process of atherosclerosis (38). In addition, H_2O_2 is a crucial component of the redox signaling cascade which is involved in smooth muscle cell proliferation and migration in atherosclerosis (39). Additionally, the autophagy caused by H_2O_2 also contributes to atherosclerosis (40).

The findings of the present study demonstrate that H_2O_2 treatment of macrophage cells results in significantly increased ANGPTL4 expression and that the p38 MAPK and ERK1/2 signaling pathways are involved in the process. However, further studies are required to explain the exact effect of H_2O_2 on ANGPTL4 expression in atherosclerosis. Whether H_2O_2 acts as a trigger to activate other inflammatory factors that consequently promote ANGPTL4 expression is unknown. In addition, the inhibitors of p38 MAPK and ERK1/2 have not blocked H_2O_2 induced ANGPTL4 protein release completely, and whether other signaling pathways such as the PPARs pathways are implicated in this process requires further research. The present study reveals the communication between H_2O_2 and ANGPTL4 for the first time, and their interaction may produce an effect on atherosclerosis.

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