Paeoniflorin inhibits proliferation of endometrial cancer cells via activating MAPK and NF-κB signaling pathways

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Abstract. Paeoniflorin (PAE), a principal bioactive component of *Paeonia lactiflora* Pall., appears to have antitumor properties. However, the pharmacological activity of PAE in endometrial cancer and the specific mechanisms have remained largely elusive. The present study aimed to determine the antitumor activity of PAE in the human endometrial cancer cell line RL95-2 and explore the potential mechanisms. Cell proliferation was assessed to evaluate the antitumor effect of PAE towards RL95-2 cells via a Cell Counting Kit-8 assay. Protein expression was examined to investigate changes in the signaling pathways of p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and nuclear factor (NF)-κB in RL95-2 cells during PAE treatment by western blot analysis. The results revealed that PAE significantly and dose- and time-dependently inhibited the proliferation of RL95-2 cells. In addition, PAE activated MAPK signaling pathways (p38, JNK and ERK) and the NF-κB signaling pathway. Furthermore, p38 MAPK and NF-κB inhibitors (SB203580 and MG-132, respectively) prevented PAE-induced proliferative inhibition in RL95-2 cells. However, ERK and JNK inhibitors (PD98059 and BI-78D3, respectively) did not produce such an inhibition. In conclusion, the present study demonstrated that PAE exerts its anti-proliferative activity via activating p38 MAPK and NF-κB signaling pathways in endometrial cancer cells, providing a potential new drug of choice for endometrial cancer therapy.

Introduction

In western countries, endometrial cancer is well known as one of the most common gynecological cancers and in Asian countries, the morbidity has been increasing (1). The incidence of endometrial cancer has had annual increases of 1.9% among women ≥50 years and 1.3% among women <50 years of age. The mortality has been multiplying by 1.1% per year and for 2017, ~10,585 deaths are anticipated (2). At present, the primary standard treatment for endometrial cancer is surgery. Chemotherapy, radiation therapy or their combination is chosen for patients with a medium or high recurrence risk. Most endometrial cancer patients were diagnosed in the early stage and treated with surgery alone or with additional radiation therapy (3). However, a significant proportion of endometrial cancer patients with metastasis or in the advanced stage still require systematic chemotherapy (4,5). Taxanes, platinum and adriamycin alone or in combination are currently used for recurrent or progressing endometrial cancer patients. The response rate of adriamycin and cisplatin combination in advanced endometrial carcinoma patients is up to 42% (6). However, the search for effective agents against either recurrent or advanced endometrial cancer remains disappointing (7). Accordingly, the development of novel compounds against endometrial cancer has attracted great attention and continuous research efforts are being made towards this goal. However, only a small number of studies on novel compounds with activity against endometrial cancer are currently available and the mechanisms remain largely elusive.

Novel antitumor agents derived and identified from natural sources have attracted exponential interest. The compounds represent a key alternative for the improvement of existing standard cancer therapies (8,9). Paeoniflorin (PAE) is a principal bioactive component of the root of *Paeonia lactiflora* Pall., a medicinal herb species found in Korea, Japan and China (10). In Traditional Chinese Medicine, PAE has been widely used (11). It has been reported that PAE exerts numerous pharmacological effects, such as anti-inflammatory (12,13), anti-allergy (14,15), immunoregulatory (16,17), neuroprotective (18) and hepatoprotective effects (19). Recent studies have suggested that PAE may be a novel potential antitumor agent. PAE inhibits the proliferation of a variety of human cancer cell types, including gastric carcinoma (10,11), lung cancer (19) and hepatocellular carcinoma cells (20). PAE was reported to induce cell cycle arrest via activating p53/14-3-3 zeta in HT29 colorectal cancer cells (21) and to promote apoptosis by downregulating matrix metalloproteinase-9 and upregulating microRNA-16 in human

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glioma cells (22). However, the anti-cancer effects of PAE on endometrial cancer have remained indeterminate.

In the present study, RL95-2 human endometrial cancer cells were selected to investigate the potential effect of PAE on the inhibition of cell proliferation. In addition, changes of protein expression in the mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) signaling pathways as underlying mechanisms of PAE-mediated antitumor activity were studied. According to these attributes, PAE may be a promising novel candidate compound for the treatment of endometrial cancer.

Materials and methods

Reagents. Paeoniflorin (purity, ≥98%) and 5-fluorouracil (5-FU) were obtained from Aladdin Biochem Technology Co., Ltd. (Shanghai, China). Cell Signaling Technology, Inc. (Beverly, MA, USA) supplied the inhibitors SB203580, PD98059 and MG-132. Wuxi Jinpu Bio-Technology Co., Ltd. (Jiangsu, China) provided the inhibitor BI-78D3. The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan).

Cell culture. The Cell Bank of the Chinese Academy of Sciences (Shanghai, China) supplied the RL95-2 human endometrial carcinoma cell line, and the HECCL-1 human endometrial carcinoma cell line was purchased from BeNa Culture Collection (Suzhou, China). Cells were cultured in Dulbecco's modified Eagle's medium/F12 (HyClone; GE Healthcare, Little Chalfont, UK) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml streptomycin and 100 U/ml penicillin (Beyotime Institute of Biotechnology, Inc., Haimen, China) at 37˚C in a humidified atmosphere containing 5% CO_2. A total of 2 h prior to the penicillin process, 50 μM SB203580, PD98059, BI-78D3 and MG-132 (Sigma-Alrich; Merck KGaA; Darmstadt, Germany) were pre-incubated with RL95-2 for 1 h at room temperature.

Cell proliferation assay. RL95-2 cells were seeded into 96-well plates at $1x10^4$ cells/well and treated for the assigned times with either vehicle (0.1% dimethyl sulfoxide) or PAE at the indicated concentrations. The CCK-8 assay was performed to assess cell proliferation according to the manufacturer's instructions. The numerical absorption values obtained with an ELISA plate reader (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm were used to evaluate the cell viability.

Western blot analysis. RL95-2 cells were treated and the lysate was prepared as described previously (23). The protein (20 μg) was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore; Billerica, MA, USA). Immunoblots were exposed to primary antibodies at room temperature for 1 h. Antibodies used were against p38 (9212; 1:2,000 dilution), phosphorylated (p)-p38 (4511l 1:1,000 dilution), NF-κB p65 (8242l 1:1,500 dilution), p-p65 (3033l 1:2,000 dilution), extracellular signal-regulated kinase (ERK; 4695; 1:1,000 dilution), p-ERK (4370l 1:1,500 dilution) (all from Cell Signaling Technology, Inc.), c-Jun N-terminal kinase (JNK; ab179461; 1:1,000 dilution) or p-JNK (ab76572; 1:1,500 dilution) (all from Abcam, Cambridge, MA, USA). Subsequently, blots were incubated at room temperature for 40 min with horseradish peroxiase-conjugated secondary antibodies (BA1054; 1:20,000 dilution; Boster Biological Technology, Wuhan, China). The bands with immunoreactive proteins on the membrane were assessed by the ECL Plus Western Blotting Detection system (EMD Millipore). GAPDH was selected as the reference protein (kC-5G5; 1:10,000 dilution; Kangchen Biotech Co., Ltd., Shanghai, China).

Statistical analysis. Data analysis was performed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). All values are expressed as the mean ± standard deviation. Student's t-test was applied to assess differences between two samples. Multiple comparison tests were performed via one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PAE inhibits human endometrial cancer cell proliferation. The effect of PAE on human endometrial cancer cell proliferation was initially assessed in RL95-2 cells. As depicted in Fig. 1A, PAE treatment at 100, 200, 400 and 800 μg/ml for 24 h resulted in a marked inhibition of proliferation in a dose-dependent manner. 400 μg/ml PAE exerted significant anti-proliferative activity ($P<0.001$) and was chosen to assess the role of time in PAE-induced proliferative inhibition. As displayed in Fig. 1B, treatment with 400 μg/ml PAE for 24, 48 and 72 h inhibited cell proliferation in a time-dependent manner. Similar results were obtained in anther endometrial cancer cell line, HECCL-1 (Fig. 1C).

Roles of MAPK signaling pathways in PAE-induced proliferative inhibition

Role of p38 MAPK in PAE-induced proliferative inhibition. Following vehicle or PAE treatment of RL95-2 cells for 24 h, the levels of p38, p-p38, p65 and p-p65 were detected by western blot analysis. PAE significantly upregulated p-p65 and p-p38 ratio ($P<0.05$, $P<0.01$; Fig. 2A and B), suggesting that PAE activated the p38 MAPK signaling pathway in RL95-2 cells. To clarify the role p38 MAPK activation in PAE-mediated proliferative inhibition, the p38 MAPK inhibitor SB203580 was added. Addition of SB203580 abolished the increase in p-p65 and p-p38 and prevented the proliferative inhibition induced by PAE ($P<0.01$; Fig. 2C). These findings suggested that the p38 MAPK signaling pathway may have an important role in the anti-proliferative effect of PAE on RL95-2 cells.

Role of ERK in PAE-induced proliferative inhibition. Next, the effect of ERK in the proliferative inhibition induced by PAE was evaluated. As depicted in Fig. 3A and B, PAE treatment caused a significant increase of p-ERK and p-p65 levels, which was significantly inhibited by ERK inhibitor PD98059. However, in the proliferation assay, pre-treatment with PD98059 did not affect the PAE-induced proliferative inhibition ($P>0.05$; Fig. 3C).

Role of JNK in PAE-induced proliferative inhibition. As illustrated in Fig. 4, PAE treatment caused a marked increase of

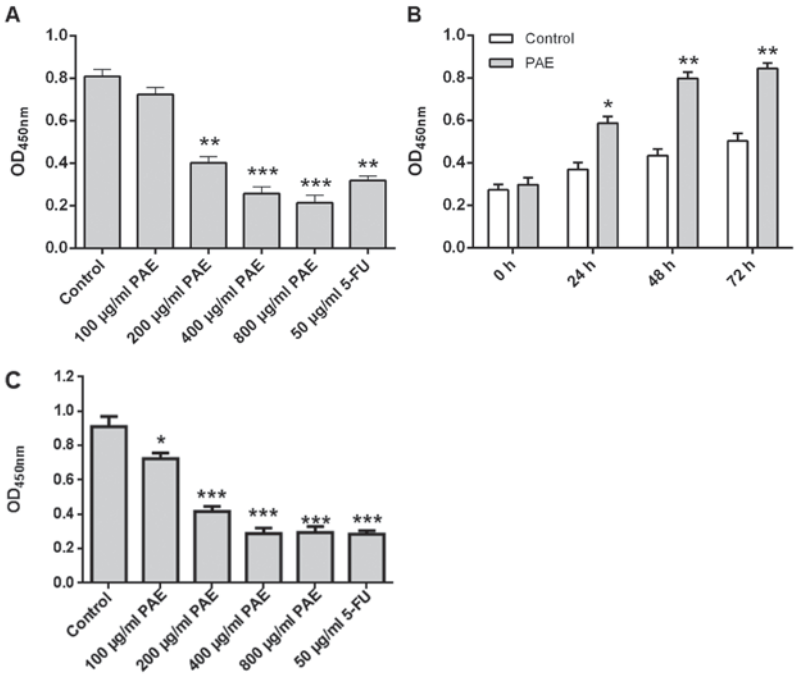

Figure 1. Effect of PAE on the proliferation of RL95-2 cells. (A) RL95-2 cells were incubated overnight, exposed to PAE (100, 200, 400 and 800 μg/ml) or 50 μg/ml 5-FU and then cultured for 24 h. (B) Cells were incubated overnight, exposed to 400 μg/ml PAE and then cultured for 24, 48 and 72 h. (C) HECCL-1 cells were incubated overnight, exposed to PAE (100, 200, 400 and 800 μg/ml) or 50 μg/ml 5-FU and then cultured for 24 h. Inhibition of proliferation was measured via the Cell Counting Kit-8 assay. Values are expressed as the mean ± standard deviation (n=3). ***P<0.001, **P<0.01 and *P<0.05 vs. control. PAE, paeoniflorin; 5-FU, 5-fluorouracil; OD450, optical density at 450 nm.

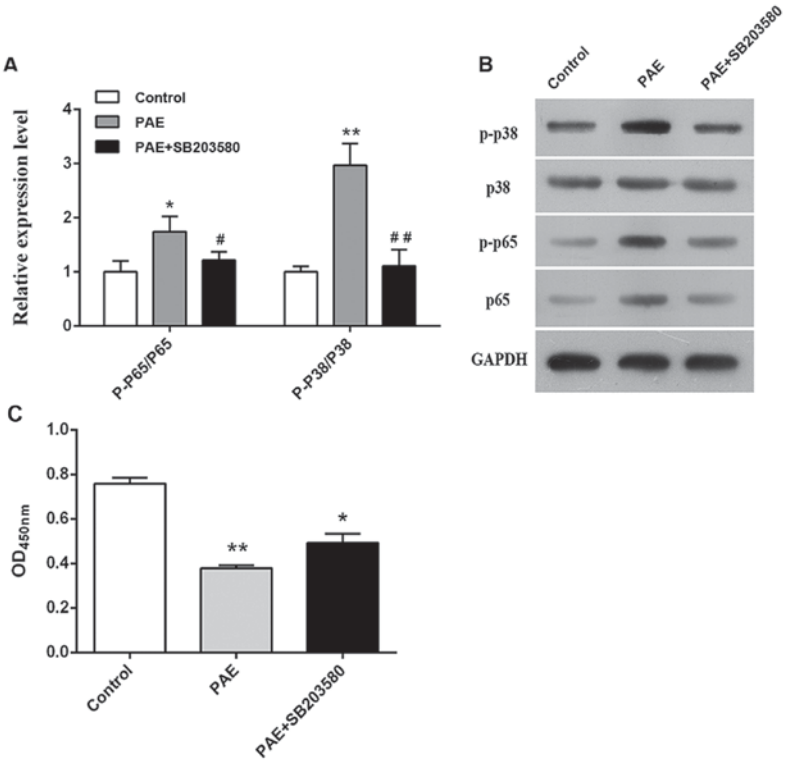

Figure 2. Role of p38 mitogen-activated protein kinase in PAE-induced proliferative inhibition. (A and B) RL95-2 cells were treated with vehicle, 400 μg/ml PAE or 400 μg/ml PAE + SB203580 for 24 h, and p38, p-p38, p65 and p-p65 levels were detected by western blot analysis. (C) Cells were exposed to vehicle, 400 μg/ml PAE or 400 μg/ml PAE + SB203580 for 24 h and cell viability was measured via the Cell Counting Kit-8 assay. Values are expressed as the mean ± standard deviation (n=3). **P<0.01 and *P<0.05 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. PAE group. PAE, paeoniflorin; p-p38, phosphorylated p38; OD450, optical density at 450 nm.

p-JNK levels compared with those in the control group. JNK inhibitor BI-78D3 significantly abolished the PAE-induced increase in p-JNK expression. However, BI-78D3 pre-treatment did not significantly affect the anti-proliferative effect

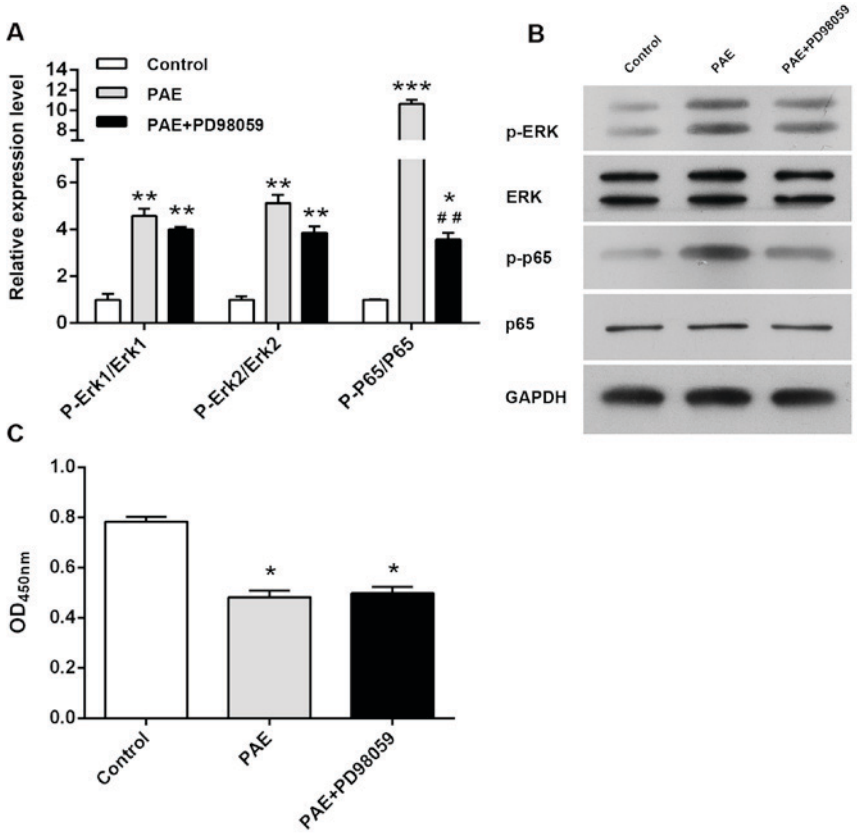

Figure 3. Role of ERK in PAE-induced proliferative inhibition. (A and B) RL95-2 cells were treated with vehicle, 400 μg/ml PAE or 400 μg/ml PAE + PD98059 for 24 h, and ERK, p-ERK, p65 and p-p65 levels were detected by western blot analysis. (C) Cells were exposed to vehicle, 400 μg/ml PAE or 400 μg/ml PAE + PD98059 for 24 h and cell viability was measured via the Cell Counting Kit-8 assay. Values are expressed as the mean ± standard deviation (n=3). ***P<0.001, **P<0.01 and *P<0.05 vs. control; $^{\#\#}$P<0.01 vs. PAE group. PAE, paeoniflorin; p-p65, phosphorylated p65; OD450, optical density at 450 nm; ERK, extracellular signal-regulated kinase.

of PAE, suggesting that JNK inhibition did not interfere with the ability of PAE to inhibit cell proliferation (P>0.05, Fig. 4).

Role of NF-κB signaling pathway in PAE-induced proliferative inhibition. The present study further examined the effect of PAE on NF-κB p65 and p-p65 levels in order to explore the role of NF-κB in the PAE-mediated proliferative inhibition. PAE treatment of RL95-2 cells resulted in a marked upregulation of p-p65 levels (P<0.05, P<0.01; Fig. 5A and B), suggesting that PAE activated the NF-κB signaling pathway. It was then assessed whether NF-κB activation had a role in PAE-induced proliferative inhibition. Pre-treatment with the NF-κB inhibitor MG-132 attenuated the increase in p-p65 expression. In addition, MG-132 pre-treatment yielded a significant suppression of PAE-induced proliferative inhibition (P<0.05; Fig. 5C). These observations indicated that the anti-proliferative activity of PAE against RL95-2 cells is mediated via the activation of the NF-κB signaling pathway.

Discussion

The present study aimed to investigate the anti-cancer activity of PAE in human endometrial cancer cells and the potential underlying mechanisms. The results revealed that PAE induced a significant dose- and time-dependent inhibition of endometrial cancer cell growth. As the MAPK and NF-κB signaling pathways are crucial in cancer cell proliferation, the roles of associated signaling proteins in PAE-induced antitumor activity were then explored.

The MAPK family is involved in the regulation of gene expression in response to the extracellular stimulation signals and activation of various cell responses, such as apoptosis, differentiation and cell proliferation (13). MAPKs include three key groups: p38 MAPK, JNK and ERK (24). In the present study, PAE increased the levels of phosphorylated p38 MAPK, ERK and JNK, suggesting that the p38 MAPK, ERK and JNK signaling pathways were activated during the treatment of RL95-2 cells with PAE. Furthermore, the function of MAPK signaling pathways in the anti-proliferative effects of PAE was assessed by applying p38 MAPK, ERK and JNK kinase inhibitors. Pre-treatment with p38 MAPK inhibitor effectively prevented PAE-induced proliferative inhibition, whereas ERK and JNK kinase inhibitors did not. This result suggested that PAE inhibits the proliferation of RL95-2 cells through the p38 MAPK signaling pathway, but not through the ERK and JNK pathways.

p38 MAPK has been reported to have a critical role in various cancer types, including prostate (25), breast (26) and liver cancer (27). MAPK inhibitors may be promising drugs against solid tumors of lung, liver, bladder, breast and prostate (28). It has been suggested that PAE may act as an antitumor agent against other solid tumor types via the p38 MAPK signaling pathway. In fact, PAE exerts antitumor activities against various solid tumor types, including gastric

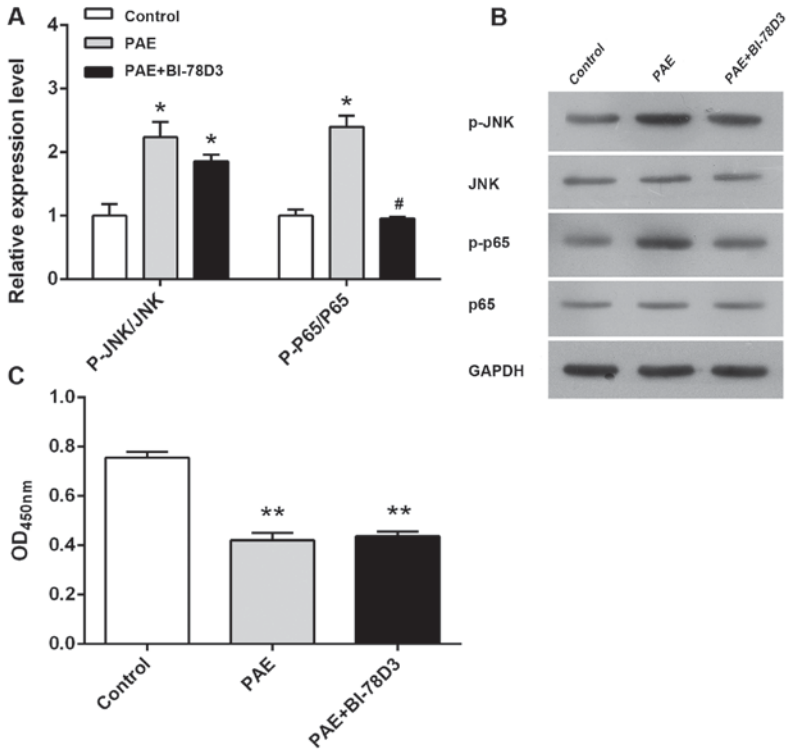

Figure 4. Role of JNK in PAE-induced proliferative inhibition. (A and B) RL95-2 cells were treated with vehicle, 400 μg/ml PAE or 400 μg/ml PAE + BI-78D3 for 24 h, and JNK, p-JNK, p65 and p-p65 levels were detected by western blot analysis. (C) Cells were exposed to vehicle, 400 μg/ml PAE or 400 μg/ml PAE + BI-78D3 for 24 h and cell viability was measured via the Cell Counting Kit-8 assay. Values are expressed as the mean ± standard deviation (n=3). **P<0.01 and *P<0.05 vs. control; $^{\#}$P<0.05 vs. PAE group. PAE, paeoniflorin; p-p65, phosphorylated p65; OD450, optical density at 450 nm; JNK, c-Jun N-terminal kinase.

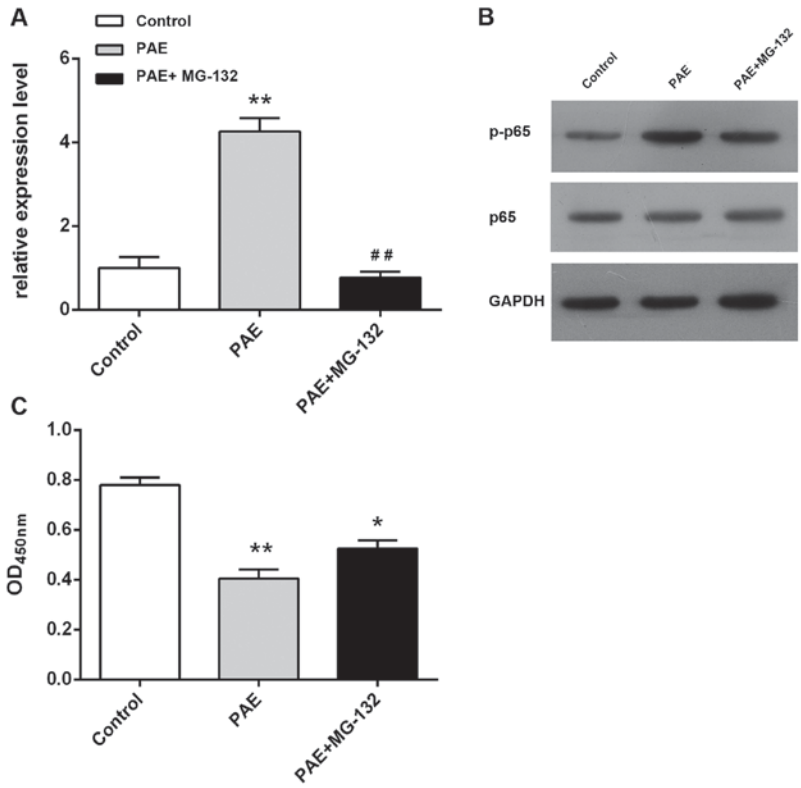

Figure 5. Role of NF-κB signaling pathway in PAE-induced proliferative inhibition. (A and B) PAE activated NF-κB signaling pathway. RL95-2 cells were treated with vehicle, 400 μg/ml PAE or 400 μg/ml PAE+ MG-132 for 24 h and p65 and p-p65 levels were detected by western blot analysis. (C) MG-132 prevented the proliferative inhibition induced by PAE. Cells were incubated overnight, exposed to vehicle, 400 μg/ml PAE or 400 μg/ml PAE + MG-132 for 24 h and cell viability was measured via the Cell Counting Kit-8 assay. Values are expressed as the mean ± standard deviation (n=3). **P<0.01 and *P<0.05 vs. control; $^{\#\#}$P<0.01 vs. PAE group. PAE, paeoniflorin; p-p65, phosphorylated p65; OD450, optical density at 450 nm; NF, nuclear factor.

carcinoma (10,11), lung cancer (19), liver cancer (20), while the involvement of the p38 MAPK pathway in its antitumor activities requires further investigation.

NF-κB, a heterodimeric complex composed of proteins p65 and p50, is an important transcriptional regulatory factor. It inhibits cell metastasis, invasion, cell cycle, apoptosis and cell proliferation by regulating a variety of target genes (29). In the present study, PAE was found to increase the levels of p-p65, suggesting that PAE activates the NF-κB signaling pathway. Furthermore, pre-treatment with NF-κB inhibitor partially inhibited PAE-induced proliferative inhibition in RL95-2 cells. As NF-κB is one of the substrates of the MAPKs signaling pathways and is therefore correlated with them, the present study also examined the effect of MAPK inhibitors on the levels of p-p65. The results demonstrated that all MAPK inhibitors decreased the levels of p-p65, but only p38 MAPK inhibitor prevented PAE-induced proliferative inhibition. These findings suggested that PAE inhibits RL95-2 cell proliferation partially via the NF-κB signaling pathway.

NF-κB has a key role in inflammatory and immune regulation and is found in a diversity of cells (30). Zhang *et al* (12) reported that PAE revokes colitis induced by dextran sulfate sodium through the Toll-like receptor 4-dependent pathway. In rats with adjuvant arthritis, Wu *et al* (16) found that PAE caused immune tolerance by enhancing the desensitization of β2-adrenergic receptor. These studies demonstrated that PAE displays anti-inflammatory or immuoregulatory activities, whereas the involvement of NF-κB in these processes requires further study.

Of note, emerging evidence has indicated a positive correlation between NF-κB activation and cancer progression in a variety of tumor types (31,32). In human gastric cancer, Wu *et al* (8) found that PAE restrains NF-κB activation and promotes the apoptosis induced by 5-FU. Furthermore, Fang *et al* (10) reported that PAE modulates multidrug resistance of SGC7901/vincristine by effectively inhibiting the activation of NF-κB. Furthermore, PAE inhibited NF-κB activation by significantly decreasing p65 expression and inhibiting intra-nuclear p65 transcription activation (10). These studies appear to contradict the findings of the present study. However, the dual effects of PAE on NF-κB occurred under different circumstances, indicating that PAE may be a potential drug candidate for treating cancer, inflammation and immunological diseases via bidirectional modulation of the NF-κB signaling pathway.

The findings of the present study therefore confirmed that PAE inhibits endometrial cancer cell proliferation via activating the MAPK and NF-κB signaling pathways, which may also be associated with the anti-inflammatory and immunoregulatory activities of PAE. For the treatment of endometrial cancer, targeting these signaling pathways mat provide an efficient alternative approach. The present study is expected to provide a foundation for future study on endometrial cancer therapy and more extensive applications of PAE.

In conclusion, the present study demonstrated that PAE, a principal bioactive component of *Paeonia lactiflora* Pall., significantly inhibited the proliferation of endometrial cancer cells, thereby providing a potential novel approach for clinical endometrial cancer treatment. Furthermore, the antitumor effect of PAE was found to be mediated via the activation of p38 MAPK and NF-κB signaling pathways, which is likely linked to the anti-inflammatory and immunoregulatory activities of PAE.

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