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The p-ERK–p-c-Jun–cyclinD1 pathway is involved in proliferation of smooth muscle cells after exposure to cigarette smoke extract

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ABSTRACT

An epidemiological survey has shown that smoking is closely related to atherosclerosis, in which excessive proliferation of vascular smooth muscle cells (SMCs) plays a key role. To investigate the mechanism underlying this unusual smoking-induced proliferation, cigarette smoke extract (CSE), prepared as smoke-bubbled phosphate-buffered saline (PBS), was used to induce effects mimicking those exerted by smoking on SMCs. As assessed by Cell Counting Kit-8 detection (an improved MTT assay), SMC viability increased significantly after exposure to CSE. Western blot analysis demonstrated that p-ERK, p-c-Jun, and cyclinD1 expression increased. When p-ERK was inhibited using U0126 (inhibitor of p-ERK), cell viability decreased and the expression of p-c-Jun and cyclinD1 was reduced accordingly, suggesting that p-ERK functions upstream of p-c-Jun and cyclinD1. When a c-Jun over-expression plasmid was transfected into SMCs, the level of cyclinD1 in these cells increased. Moreover, when c-Jun was knocked down by siRNA, cyclinD1 levels decreased. In conclusion, our findings indicate that the p-ERK–p-c-Jun–cyclinD1 pathway is involved in the excessive proliferation of SMCs exposed to CSE.

1. Introduction

Atherosclerosis is a major cause of morbidity and mortality [1,2]. Atherosclerosis is characterized by excessive proliferation of vascular smooth muscle cells (SMCs), and is the consequence of a combination of many factors [3,4].

One of the proteins involved in cell proliferation is extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) subfamily. The ERK pathway plays a role in many biological processes besides cell proliferation [5–7]. Moreover, it plays an important role in transferring extracellular signals into the intracellular environment to certain transcription factors, the internal signal cell transfer station. As an important transcription factor, c-Jun is activated by phosphorylation (p-c-Jun), and then induces expression of many proteins associated with cell proliferation [8]. CyclinD1, which is a regulatory protein of the cell cycle and has been studied extensively in many researches [9], plays a key role in growth control and G1/S transition.

In this study, we speculated that there may be a relationship between smoking and excessive proliferation of SMCs in atherosclerosis. Our results confirmed that there is a relationship between cigarette smoke extract (CSE) and SMC proliferation, and that expression of p-ERK, p-c-Jun, and cyclinD1 is involved in the molecular mechanism underlying the excessive proliferation of SMCs exposed to CSE.

2. Materials and methods

2.1. Cell lines and culture

Rabbit aortic SMCs (Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College) were chosen to be the subject of the study. The cells were cultured in DMEM/high-sugar basal medium (Hyclone, Logan, Utah, USA) with 10% newborn calf serum (Gibco, Carlsbad, CA, USA).

2.2. Generation of cigarette smoke extract

Filtered cigarettes (tar content: 10 mg, nicotine content: 0.9 mg, smoke gas [CO]: 12 mg) were smoked using a pump. Six cigarettes

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were bubbled through 500 ml phosphate-buffered saline (PBS), and this solution was defined as the original CSE. The pH of the CSE was adjusted to 7.4, and the solution was filtered through a 0.22-μm filter (Roche, Basel, Switzerland). The same batch of PBS, but which had not been smoke-bubbled, was used as control. CSE was aliquoted and stored at −80 °C until required for use [10,11].

2.3. Determination of cell viability

After digesting cells in the logarithmic growth phase and counting the amount of cells, cells were seeded into 96-well plates at 3000 cells per well, and cultivated in 5% CO_2, in a 37 °C incubator for 24 h. Then, medium was replaced with fresh medium containing CSE of different concentrations, with or without 10 μM U0126 (Cell Signaling Technology, Boston, MA, USA), which was used to inhibit expression of p-ERK. As a control, corresponding concentrations of PBS were added to cells. Cells were then cultivated in 5% CO_2, at 37 °C in an incubator for 48 h. The medium was then removed and replaced with fresh medium containing 10% Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) [12], and the cells cultivated in 5% CO_2, at 37 °C in an incubator for 2 h; in addition, blank wells were set up that contained medium with 10% CCK-8 only. Absorbance values were measured at 450 nm. Cell viability, which represents proliferation, was calculated with the formula Cell viability = [A (CSE) − A (blank)]/[A (PBS) − A (blank)]. All experiments were performed in triplicate.

2.4. Plasmid transfection

The plasmid over-expressing c-Jun used in this study (V5-c-Jun) was kindly provided by the National Laboratory of Medical Molecular Biology (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College). Cells were grown in 6-cm culture dishes until 80% confluent. Then, 180 μl DMEM/high glucose basal medium, to which 3 μg of the plasmid had been added, was mixed with 180 μl DMEM/high glucose basal medium containing 5 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA); the mixture was then left to stand for 15 min. The medium was removed from the cells, which were then washed twice with DMEM/high-sugar basal medium, after which the cells were cultured in 2 ml DMEM/high-sugar basal medium, to which the lipofectamine–plasmid complex had been added, for 6 h, in 5% CO_2, at 37 °C in an incubator.

2.5. siRNA interference

c-Jun siRNA (sense, 5′-AAGAACUCGGACCUCCUCACC-3′) and negative control siRNA (sense, 5′-UUCUCCGAACGUGUCACGU-3′) oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were incubated in 6-well plates until 80% confluent; the medium was then replaced with 1 ml fresh DMEM/high glucose complete medium. Transfection was performed with Lipofectamine RNAiMAX Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), after which the cells were incubated in 5% CO_2, at 37 °C for 24 h.

2.6. Western blotting

Proteins extracted from cells were separated by SDS–PAGE, and then electrophoretically transferred to a polyvinylidene fluoride membrane. Specific proteins were detected using the following antibodies from Cell Signaling Technology (Danvers, MA, USA): anti-ERK rabbit monoclonal antibody (mAb; 137F5); anti-phospho-ERK (Thr202/Tyr204), anti-c-Jun, anti-p-c-Jun (Ser63), anti-JNK rabbit mAb (56G8), anti-phospho-JNK (Thr183/Tyr185), anti-cyclinD1, and anti-tubulin.

Bovine serum albumin (BSA, 5%) in Tris-based buffer (TBS)–Tween20 was used as blocking and washing solution. Horseradish peroxidase-conjugated secondary antibodies were then incubated with the blot, and antibody complexes were detected by chemiluminescence (Engreen, Beijing, China).

2.7. Statistical analysis

All data are expressed as mean ± standard error and were compared by ANOVA (using SPSS17.0). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Schematic diagram of CSE-producing device

As shown in Fig. 1, the device was designed to produce CSE. A burning cigarette was placed into the combustion chamber, which had a plughole to allow air inflow. The combustion chamber not only provided the place in which to burn the cigarette, but also collected the cigarette smoke. After imposing a negative pressure to the right of this chamber, the cigarette smoke passes through the cigarette itself and into the gas chamber, as it would when a person smokes a cigarette. The valve between the combustion chamber and the gas chamber allows control of the amount of airflow to imitate the actual smoking process, and the valve could be removed in order to introduce a new cigarette. The gas chamber, with a nonporous plug, acted as smoke container to reduce airflow, so that the smoke could be fully dissolved in the PBS in the conical flask. On the right, there are 2 test tubes containing sodium hydroxide solution, which isolates the chamber from the outside air, provides a sterile environment, and cleans exhaust gases maximally. The right port connects to a negative pressure-inducing suction device. Throughout the device, PBS was maintained in a sterile environment. The CSE obtained by the device strongly smelled of cigarette smoke.

3.2. CSE can induce proliferation of SMCs

As shown in Fig. 2A, SMC proliferation was higher when the cells were cultured in the presence of CSE, with concentrations ranging from 1.25% to 20%. SMC viability was lower in the presence of low as well as high concentrations of CSE: it had no proliferating effect at concentrations below 1.25%, and proved toxic at high concentrations (>20%).

The reasons for SMC proliferation may include decreased degradation of cell cycle proteins [13], proliferation signaling pathway [14], et al. It is known that MAPKs play an important role in transferring extracellular signals to the intracellular environment; the ERK pathway is known to be involved in cell proliferation [15] and in many changes (activation or degradation) in proteins in the signaling pathway. We therefore investigated whether ERK was involved in the increased SMC viability. After adding the p-ERK-inhibitor U0126, SMC viability was reduced (Fig. 2A). Thus, CSE could induce SMC proliferation via a pathway involving p-ERK. A CSE concentration of 5%, which fell in the center of the plateau phase (Fig. 2A), was chosen as the working concentration for subsequent experiments (Fig. 2C). This concentration was equivalent to consumption of 9 cigarettes a day [16].

There was no difference in the expression of ERK between the 5%CSE groups and the 5%PBS groups ($p > 0.05$; Fig. 2B), indicating that U0126 had no effect on ERK *per se*, as indicated by the manufacturer; in contrast, the expression of p-ERK increased

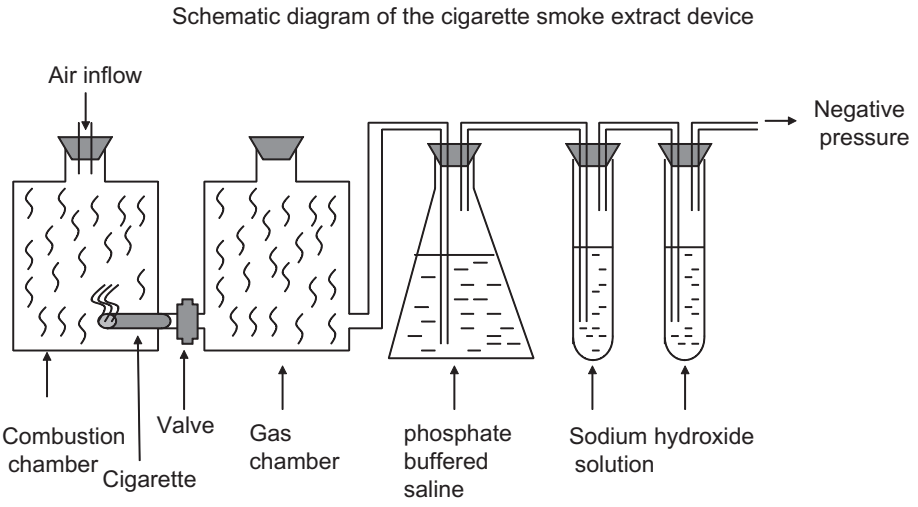

Fig. 1. Schematic diagram of the cigarette smoke extract device.

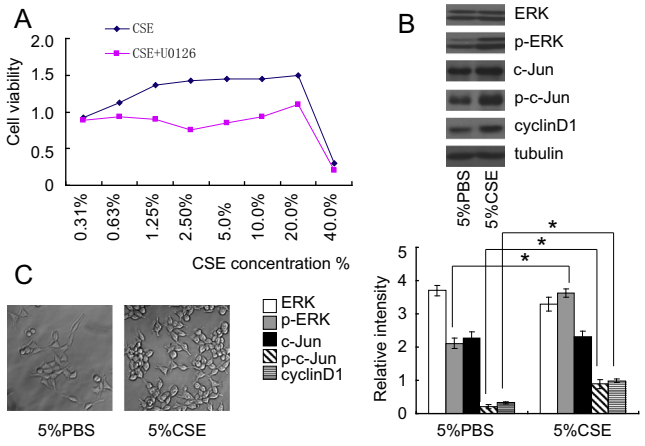

Fig. 2. The effect of cigarette smoke extract (CSE) on the viability of smooth muscle cells (SMCs) and the expressions of signaling proteins. (A) The viability of SMCs exposed to different concentrations of CSE in the presence or absence of U0126. The horizontal axis indicates the concentration of CSE contained in the medium. *CSE group*: SMCs were incubated in medium containing CSE for 48 h. *CSE + U0126 group*: SMCs were incubated in medium containing CSE and U0126 (10 μM) for 48 h. (B) We chose 5% as the working CSE concentration; this concentration falls in the center of the plateau phase shown in Fig. 2A. This concentration was equivalent to consumption of 9 cigarettes a day. A concentration of 5% phosphate-buffered saline (PBS) was used as control. The two groups were defined as the *5%PBS group* and *5%CSE group*. SMCs were exposed to 5%CSE or 5%PBS for 12 h. (C) Light microscopy images demonstrated that the number of cells exposed to CSE increased as compared to the 5%PBS control. SMCs were separated into two groups from one dish. *5%PBS group*: SMCs were cultured in the presence of 5% PBS for 48 h, as control; *5%CSE group*: SMCs were cultured in the presence of 5% CSE for 48 h. $^{*}p < 0.05$.

significantly after exposure to 5% CSE ($p < 0.05$), suggesting that the ERK pathway was activated upon exposure of cells to CSE. Taken together, these results (Fig. 2A and B) indicated that the effects of CSE on SMCs were associated with activation of the ERK pathway and increased p-ERK expression.

As a transcription factor, c-Jun can regulate the expression of many genes [17]. c-Jun can transduce a variety of signals from the external environment, and is activated by phosphorylation, which then lead cells to adapt to external stimuli. It has been reported that c-Jun plays a positive role in angiogenesis [18]. Therefore, it is reasonable to speculate that c-Jun may act as an index of proliferation of SMCs after exposure to CSE. There was no difference in c-Jun expression between the 5%CSE group and 5%PBS group ($p > 0.05$; Fig. 2B), while the expression of p-c-Jun in the 5%CSE group was higher than that in the 5%PBS group ($p < 0.05$). This indicated that CSE can influence the phosphorylation of c-Jun, rather than expression of c-Jun itself.

The cell viability experiments and microscope images proved that CSE can promote SMC proliferation (Fig. 2A and C). As p-c-Jun functions as a transcription factor, it may improve the expression of proteins related to cell proliferation. CyclinD1, which is thought to be a key regulator of growth control and G1/S transition in the cell cycle, is reported to be involved in SMC proliferation [19,20]. Thus, it seemed reasonable to investigate whether cyclinD1 is involved in proliferation of SMCs after exposure to CSE. In this study, cyclinD1 expression was found to be increased in SMCs after exposure to CSE; its expression was higher in the 5%CSE group than in the 5%PBS group ($p < 0.05$, Fig. 2B).

Taken together, the proliferation of SMCs induced by CSE is associated with an increase in the expression of p-ERK, p-c-Jun, and cyclinD1.

3.3. U0126 can inhibit the effects of CSE on SMCs

The experiment shown in Fig. 3 was designed to further investigate the relationship among p-ERK, p-c-Jun, and cyclinD1 in SMCs exposed to CSE. As the central signaling molecule in the ERK pathway, ERK was stably expressed in the 4 groups of cells, and its expression was not different in the control versus the U0126, or the CSE versus CSE + U0126 groups (all $p > 0.05$). As an inhibitor of p-ERK, U0126 specifically inhibits p-ERK levels, but had no effect on expression of ERK itself. When comparing the U0126 group with the control group, the p-ERK levels were significantly inhibited ($p < 0.05$), as expected. Moreover, the CSE + U0126 group also demonstrated lower p-ERK levels than did the CSE group ($p < 0.05$). Taken together with the results shown in Fig. 2B, these findings indicated that CSE can increase p-ERK levels, an effect that can be reduced by U0126.

As with ERK, c-Jun was also stably expressed in the 4 groups, with no significant differences between the control versus U0126, or CSE versus CSE + U0126 groups ($p > 0.05$). This may be because c-Jun is a transcription factor and U0126 is an inhibitor of p-ERK; U0126 did not affect c-Jun expression. We therefore investigated the possible effector molecules, p-c-Jun and cyclinD1, and found similar results as for p-ERK. U0126 also inhibited the expression of p-c-Jun and cyclinD1 (control versus U0126,

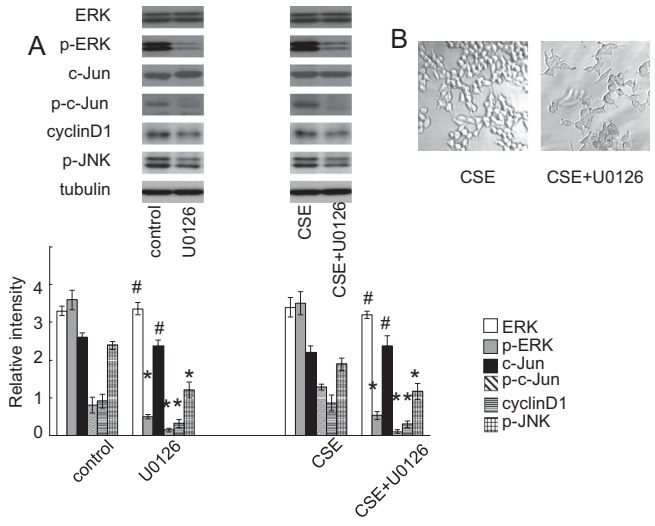

Fig. 3. Expression of factors involved in smooth muscle cell (SMC) proliferation in response to cigarette smoke extract (CSE). (A) *Control group*: SMCs were cultured in standard medium. *U0126 group*: SMCs were cultured in the presence of 10 μM U0126 for 2 h. *CSE group*: SMCs were cultured in the presence of 5% CSE for 1 h. *CSE + U0126 group*: SMCs were pre-incubated in the presence of 10 μM U0126 for 2 h, after which 5% CSE was added to the medium for 1 h. (B) Light microscopy images demonstrated that the number of cells exposed to CSE + U0126 decreased as compared to the CSE only. SMCs were separated into two groups from one dish. *CSE group*: SMCs were cultured in the presence of 5% CSE for 48 h; *CSE + u0126 group*: SMCs were cultured in the presence of 5% CSE and 10 μM U0126 for 48 h. $^{*}p < 0.05$, $^{\#}p > 0.05$.

$p < 0.05$), even in the presence of CSE (CSE versus CSE + U0126, $p < 0.05$). Given the specific inhibitory effect of U0126 on p-ERK, it is reasonable to consider that U0126 reduced the expression of p-c-Jun and cyclinD1 by means of suppressing p-ERK. In other words, p-ERK is upstream of p-c-Jun and cyclinD1 in the signaling pathway. Moreover, the expression of p-c-Jun and cyclinD1 in CSE + U0126 was also clearly inhibited by U0126 ($p < 0.05$) in comparison to the expression in the CSE group; thus, U0126 can inhibit the proliferation effect of CSE on SMCs (Fig. 3B).

We further elucidated the relationship among these signaling molecules. First, as an inhibitor of p-ERK, U0126 cannot affect the expression of p-c-Jun and cyclinD1, but we observed that the levels of these proteins were indeed reduced by U0126 in comparison to those in the control groups; this can only be the case if p-ERK is upstream of p-c-Jun and cyclinD1 in the signaling pathway. Second, when comparing CSE + U0126 with CSE groups, we found similar results to those of the U0126 versus control groups, demonstrating that, even in the presence of CSE, U0126 can also inhibit p-ERK, p-c-Jun and cyclinD1. Taken together with the findings that CSE can induce p-ERK, p-c-Jun and cyclinD1 (Fig. 2B), we deduced that the signaling pathway underlying the effect of CSE on SMCs involved p-ERK, p-c-Jun, and cyclinD1, in that order.

It is known that c-Jun can be phosphorylated by p-JNK (c-Jun-N-terminal kinase, JNK); the results above also demonstrated that changes in p-ERK levels occur prior to changes in p-c-Jun expression. Hence, we investigated the relationship between p-JNK and p-ERK. Like ERK, JNK is stably expressed in all 4 groups, with no significant differences between the control versus U0126, or CSE versus CSE + U0126 groups ($p > 0.05$), indicating that the experimental interventions had no effect on JNK itself, and only influenced its phosphorylation. Moreover, as with p-c-Jun and cyclinD1, U0126 also inhibited the expression of p-JNK (control versus U0126, $p < 0.05$), even in the presence of CSE (CSE versus CSE + U0126, $p < 0.05$). When comparing the control and U0126 groups, we found that p-JNK maybe involved in the p-ERK pathway, suggesting that p-ERK may affect p-c-Jun via p-JNK. When we compared the CSE and CSE + U0126 groups, we verified that the effect of CSE on SMC proliferation was also related to p-JNK. This implied that there is a close relationship between p-ERK and p-JNK during the process of CSE-induced SMC proliferation [21]. The details of this relationship require further exploration. Nevertheless, our results demonstrated that p-ERK can affect the expression of p-c-Jun in an as yet unknown manner.

Taken together, we found that U0126 not only inhibited p-ERK as expected, but also decreased the expression of p-c-Jun and cyclinD1. When comparing the CSE and CSE + U0126 groups, we found that U0126 can successfully and specifically inhibit the expression of p-ERK in SMCs exposed to CSE, and that U0126 can also inhibit the CSE-mediated increased expression of p-c-Jun and cyclinD1, suggesting that the pathway involved in CSE-mediated SMC proliferation involved p-ERK → p-c-Jun and p-ERK → cyclinD1. As c-Jun is a transcription factor and cyclinD1 is a cell cycle protein [22], the pathway can be simplified to p-ERK → p-c-Jun → cyclinD1.

3.4. p-c-Jun has a positive correlation with cyclinD1

In the earlier experiments, we have known p-ERK is upstream of the signaling pathway. Then, we investigated the relationship between p-c-Jun and cyclinD1 that were in the downstream part of the pathway. The expression of c-Jun and p-c-Jun was markedly higher in SMCs transfected with a plasmid over-expressing V5-c-Jun than in control cells ($p < 0.01$; Fig. 4A); moreover, the level of cyclinD1 was also higher in the transfected cells ($p < 0.01$), which indicated that there is a close relationship between c-Jun and cyclinD1. Therefore, as it is known that p-c-Jun is the activated form of c-Jun, these results suggested that p-c-Jun could indeed induce the expression of cyclinD1, supporting a p-c-Jun–cyclinD1 axis in the pathway underlying CSE-mediated SMC proliferation.

To further verify this relationship, siRNA-mediated knockdown of c-Jun was used to decrease the expression of c-Jun (Fig. 4B). The expression of c-Jun in the 3 groups treated with c-Jun siRNA were significantly lower than in the negative control (NC) siRNA group

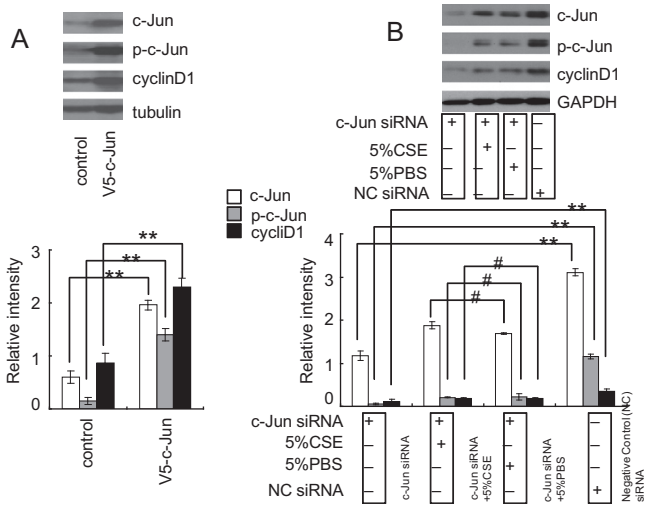

Fig. 4. Expression of cyclinD1 in the presence of increased or reduced c-Jun levels. (A) *Control group*: Smooth muscle cells (SMCs) were cultivated in standard medium. *V5-c-Jun group*: SMCs were transfected with the V5-tag c-Jun over-expressing plasmid for 6 h, and then cultivated in the standard medium for 24 h. (B) *c-Jun siRNA group*: SMCs were cultured with 20 nM c-Jun siRNA for 48 h. *c-Jun siRNA + 5%CSE group*: SMCs were cultured in the presence of 20 nM c-Jun siRNA for 24 h, after which 5% cigarette smoke extract (CSE) was added for another 24 h. *c-Jun siRNA + 5%PBS group*: SMCs were cultured in the presence of 20 nM c-Jun siRNA for 24 h, after which 5% phosphate buffered saline (PBS) was added for another 24 h. *Negative Control (NC) siRNA group*: SMCs were cultured in the presence of 20 nM NC siRNA for 48 h. $^{**}p < 0.01$, $^{\#}p > 0.05$.

($p < 0.01$), indicating that the c-Jun siRNA can successfully inhibit the c-Jun expression. Moreover, the levels of cyclinD1 decreased in accordance with the reduction of c-Jun and p-c-Jun levels in the 3 c-Jun siRNA-treated groups ($p < 0.01$). Combined with the results shown in Fig. 4A, this indicated that the pathway underlying CSE-mediated SMC proliferation involves *p*-c-Jun → cyclinD1. In addition, there was no significant difference between the c-Jun siRNA + 5%CSE and the c-Jun siRNA + 5%PBS groups in terms of the expression of p-c-Jun and cyclinD1($p > 0.05$). This indicated that, due to the reduction of c-Jun, the effects of CSE on p-c-Jun and cyclinD1 were significantly decreased, which demonstrated that the effects of CSE on SMCs rely on p-c-Jun and cyclinD1. Thus, the results shown in Fig. 4 supported involvement of a p-c-Jun–cyclinD1 signaling axis in the CSE-mediated SMC proliferation.

4. Conclusion

Smoking can lead to atherosclerosis, which features excessive proliferation of SMCs [23], suggesting a causative relationship between smoking and excessive proliferation of SMCs [24]. We demonstrated that, after exposure to certain concentrations of CSE, SMCs proliferated markedly, and p-ERK, p-c-Jun, and cyclinD1 expression increased accordingly, suggesting that these proteins maybe associated with the proliferative process. As an inhibitor of p-ERK, U0126 can inhibit these effects. Given that p-ERK can transfer extracellular signals to the intracellular environment, the pathway is likely to involve p-ERK → p-c-Jun and p-ERK → cyclinD1. When we used an over-expressing V5-c-Jun plasmid and c-Jun siRNA to increase and decrease the c-Jun expression respectively, the cyclinD1 expression changed accordingly. As c-Jun is a transcription factor and cyclinD1 is a cell cycle protein, the pathway further involved p-c-Jun → cyclinD1.

In conclusion, CSE can induce phosphorylation of ERK, and p-ERK increase p-c-Jun expression. As an activated transcription factor, p-c-Jun promotes the cyclinD1 expression. Thus, the pathway may be defined as p-ERKp-c-Jun–cyclinD1. These findings provide new insights into the signaling transduction system and the cell-cycle regulatory mechanism involved in the smoking-induced excessive proliferation of SMCs, such as smoking-related atherosclerosis. It may also provide novel clues into blocking this pathological process.

Acknowledgments

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