ARTICLE
Journal of Cellular Biochemistry 112:2082–2088 (2011)

Journal of Cellular Biochemistry

Cigarette Smoke Extract Induced Rat Pulmonary Artery Smooth Muscle Cells Proliferation via PKCα-Mediated Cyclin D1 Expression

Da-xiong Zeng,[1,2] Yong-jian Xu,[1*] Xian-sheng Liu,[1] Ran Wang,[1] and Min Xiang[1]

[1]*Key Laboratory of Pulmonary Diseases of Ministry of Health, Department of Respiratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, PR China*

[2]*Department of Respiratory Medicine, The First Affiliated Hospital of Soochow University, Suzhou 215006, PR China*

ABSTRACT

Cigarette smoke could induce pulmonary smooth muscle cells (PASMCs) proliferation. Although our previous study had implied the involvement of protein kinase Cα (PKCα), the molecular mechanism underlying PKCα pathway in this process is still unknown. In this study, rat PASMCs were stimulated by cigarette smoke extract (CSE) or PMA (a special activator to PKCα). Two percent CSE and PMA significantly enhanced cyclin D1 expression and cells proliferation. But cyclin D1-specific siRNA successfully inhibited DNA synthesis in CSE-treated or PMA-treated cells. On the other hand, PKCα-specific siRNA significantly suppressed cyclin D1 expression in CSE-treated cells. Moreover, PKCα-specific siRNA resulted in a cell-cycle arrest in G0/G1 and decreased cells number significantly. We conclude that CSE induced rat PASMCs proliferation at least partly via PKCα-mediated cyclin D1 expression. J. Cell. Biochem. 112: 2082–2088, 2011.

KEY WORDS: SMOKING; SMOOTH MUSCLE CELLS (SMCs); PROTEIN KINASE C (PKC); CYCLIN D1

Cigarette smoke induced the proliferation of pulmonary vascular smooth muscle and contributed to pulmonary hypertension [Wright et al., 2005]. It has been proved that cigarette smoke is mitogenic for vascular smooth muscle cells (SMCs) from bovine thoracic aorta, human greater saphenous vein, aortic and iliac arteries [Carty et al., 1997; Nishio and Watanabe, 1998; Li et al., 2004; Jacob et al., 2009]. But the mechanisms of its actions remain poorly understand.

The protein kinase C (PKC) family is an important intracellular mediator in signal transduction, which was responsible for a multitude of cellular processes including proliferation, differentiation, and apoptosis [Gould and Newton, 2008]. As a member of the conventional PKC subgroup, protein kinase Cα (PKCα) has been proved playing critical role in SMCs proliferation [Okazaki et al., 2000; Itoh et al., 2001]. Furthermore, our previous study implied that cigarette smoke induced rat pulmonary artery smooth muscle cells (PASMCs) proliferation via the PKCα pathway [Hu et al., 2007]. However, the downstream targets and molecular mechanism of PKCα pathway in this process is still unclear.

In the past decade, increasing evidence manifested PKC as a key regulator of cell-cycle progression [Black, 2000]. As a key regulator of G1 cell-cycle progression, cyclin D1 promotes cell-cycle progression via encoding the rate-limiting step in transitions from the G1 to S phase [Stacey, 2003]. The activation of cyclin D1 enhanced the proliferation of tumor cells [Gladden and Diehl, 2005]. Moreover, cyclin D1 has been proved playing important role in cells proliferation of SMCs from intestine, airways, and vessels [Kuemmerle et al., 2004; Pera et al., 2010; Zeng et al., 2010].

Although PKCα and cyclin D1 both acted critical role in cells proliferation, the role of PKCα in activating cyclin D1 remains controversial. An early study reported that PKCα did not exert any effect on cyclin D1 gene expression in HepG2 Cells [Marino et al., 2002]. Some studies implied that PKCα inhibited cyclin D1 expression in intestinal epithelium and prostate cancer cells [Hizli et al., 2006; Paone et al., 2008]. But, some other studies indicated that PKCα-activated cyclin D1 expression in colon cancer cells, hepatocellular carcinoma cells, and mouse skin [Garg et al., 2008; Wu et al., 2008; Gwak et al., 2009]. In our previous studies, PKCα could up-regulate cyclin D1 expression in airways SMCs from humans and asthmatic rats [Qiao et al., 2008; Du et al., 2010].

Update, the precise role of PKCα in regulating cyclin D1 expression in rat PASMCs remains unclear. So, the present study

Grant sponsor: The National Natural Science Foundation of China; Grant number: 30871128.
*Correspondence to: Dr. Yong-jian Xu, Department of Respiratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, PR China. E-mail: yjxu@tjmu.tjh.edu.cn
Received 8 November 2009; Accepted 23 March 2011 • DOI 10.1002/jcb.23131 • © 2011 Wiley-Liss, Inc.
Published online 4 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

focused on the role of PKCα on cyclin D1 expression and cell cycle in rat PASMCs exposed to CSE.

MATERIALS AND METHODS

REGENTS

Phorbol 12-myristate 13-acetate (PMA) was bought from Sigma–Aldrich Co. (USA). Mouse polyclonal antibody against 5-bromo-2-deoxyuridine (BrdU), beta-actin, and α smooth muscle actin (α-SM-actin), rabbit polyclonal antibody against PKCα and cyclin D1 were all purchased from Santa Cruz Biotechnology Inc. (USA). RT-PCR kit was product of Toyobo Biotechnology, Inc. (Japan). Lipfectine2000 and Trizol were bought from Invitrogen Biotechnology, Inc. (USA). All primers and siRNA were synthesized by Shanghai Bio-Engineering Co. Ltd (China). In Situ Cell Death Detection Kit was bought from F. Hoffmann-La Roche, Ltd (Switzerland).

PREPARATION FOR CSE SOLUTION

Cigarette smoke extract (CSE) was prepared as described previously [Zeng et al., 2010]. Smoke from four full strength Marlboro Light cigarettes was collected and passed through 100 ml of phosphate-buffered saline (PBS). This CSE solution was filtered for sterilization and then diluted using Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS). Final concentrations of the solution are expressed as (CSE solution volume/total volume) × 100%. Cells were exposed to CSE solutions for 48 h.

CELLS CULTURE

Primary rat PASMCs were prepared from explants of endothelium- and adventitia-stripped intra-pulmonary arteries of healthy Wistar Rats aged 12 weeks. Cells were cultured in DMEM with 10% FBS and identified by immunochemistry staining of α-SM-actin antibody. Passages 3–8 cells were used for all experiments. Before exposure to CSE, PMA (100 nM) or DMEM for 48 h, cells were transfected by siRNA and then starved in serum-free media. All experiments were repeated as least thrice.

SiRNA TRANSFECTION

The siRNA sequences against rat PKCα gene, cyclin D1 gene, and negative control were described in previous studies [Kundumani-Sridharan et al., 2007; Rudkouskaya et al., 2008]. Cells were 40–60% confluent when transfected. Individual siRNAs was mixed with Lipofectine2000 according to the manufacture's instructions and then applied to the cells. Efficiency of siRNA transfection was assessed by monitoring the uptake of siRNA labeled with 6-carboxyfluorescein. The transfection efficiency for each siRNA was above 90% and no significant difference existed between the three kinds of siRNA.

RNA EXTRACTION AND RT-PCR

Cells were seeded into six-well plates at a concentration of 4×10^4 cells/well. After treatment, total RNA was extracted from cells using Trizol according to the manufacture's instruction. First-strand cDNA was synthesized using reverse transcription kit. PCR reactions were carried out in thermal cycler (Bio-Rad PTC-200, USA) on a program consisting of an initial denaturation at 94°C for 5 min followed by 29 cycles of 1-min denaturation (94°C), 1-min annealing (56°C), and 1-min elongation (72°C), with a final extension period of 10 min at 72°C. Each set of PCR reactions included water as a negative control, and β-actin was used as a housekeep gene. Products of PCR reactions were size fractionated on 1.5% agarose gel and stained with 0.5 μg/ml ethidium bromide. The relative mRNA level of PKCα or cyclin D1 of each sample was determinated with the value of optical density under urtraviolet light and normalized to that of β-actin.

WESTERN BLOT

Cells were seeded into six-well plates at a concentration of 4×10^4 cells/well. Whole protein was extracted from cells after treatment. The protein content was quantified using a Bradford reagent, and then 100 μg of total protein was subjected to SDS–PAGE on 10% acrylamide gels. PKCα and cyclin D1 were detected by immunobloting with antibodies against rat PKCα (1:200) or cyclin D1 (1:500), respectively, and visualized with an enhanced chemiluminescence system. The values of optical density of visualized blots were analyzed by a gel imaging analysis system. The expression of β-actin was measured as a housekeeping gene. Visualized blots were analyzed by a gel imaging analysis system (Bio-Rad).

IMMUNOFLUORESCENCE STAINING FOR PKCα AND CYCLIN D1

Cells were seeded into 24-well plates at a concentration of 1×10^4 cells/well. After treatment, cells were fixed with cold acetone and permeabilized with 0.5% Triton X-100 in PBS for 5 min. And then cells were incubated with antibodies against PKCα (1:100) or cyclin D1 (1:100) at room temperature for 2 h. Second antibodies (Cy3-conjugated anti-rabbit IgG) were prepared in PBS and applied for 1 h at room temperature. Nuclei were stained using DAPI (5 μg/ml). Negative controls were made with PBS instead of the primary antibodies. Sections were examined using a fluorescence microscope.

CELLS PROLIFERATION AND DNA SYNTHESIS ASSAY

Cells proliferation was evaluated by cells count. Cells were seeded into six-well plates at a concentration of 4×10^4 cells/well. After treatment, cells were collected by trypsin digestion and cell numbers were calculated with a hemocytometer after trypan blue exclusion.

The BrdU incorporation was measured for DNA synthesis. After different treatment, cells were incubated with 50 mM BrdU for 4 h at 37°C and then fixed with cold acetone. Cells were permeabilized with 2% HCI and 0.5% Triton X-100 for 10 min. After incubation with the primary antibody against BrdU (1:100), the immunocytochemistry staining was applied according to the manufacture's instruction. PBS substituted for the primary antibody was used as negative control. The BrdU incorporation was expressed as the percentage of total cells.

APOPTOSIS ASSAY

Cells apoptosis was evaluated using TdT-UDP nick end labeling (TUNEL) staining as described previously [Paone et al., 2008]. After treatment, cells were fixed with 4% paraformaldehyde and then permeabilized for 30 min using 70% ethanol on ice. Nuclear DNA fragmentation was detected using an In Situ Cell Death Detection Kit

according to manufacturer instructions. The percentage of positively cells was determined by counting the numbers of labeled cells and total cells.

DNA PROFILE ANALYSIS WITH FLOW CYTOMETRY

After treatment, cells were harvested and washed twice with cold PBS, followed by fixation with 75% ethanol overnight at 4°C. The samples were subsequently stained with RNase A (50 mg/ml) and propidium iodide (50 μg/ml) for 30 min at room temperature. A FACScan machine (FACScort, BD, USA) was used to analyze the samples.

STATISTICAL ANALYSIS

All data were expressed as mean ± standard deviation and analyzed by one-way AVONA and followed by *q*-test using the computer software SPSS 12.0. For all tests, $P < 0.05$ was considered statistically significant.

RESULTS

EFFECTS OF CSE ON RAT PASMCs PROLIFERATION AND APOPTOSIS

As shown in Figure 1A,B, CSE showed a biphasic nature at low and high concentrations on cells proliferation. CSE at low concentration (such as 2%) significantly increased the cells number (by 67.62%) and BrdU incorporation (by 134.18%) as compared with control. However, CSE at high concentration (such as 20%) markedly decreased the cells number (by 45.72%) and BrdU incorporation (by 75.24%). Although 10% CSE increased cells number by 13%, there was no statistical variation as compared with control.

Moreover, we evaluated the apoptosis in CSE-treated cells. It is interesting that CSE at all concentrations did not show any significant affect on cells apoptosis. Although CSE at different concentrations slightly increased the percentage of apoptosis cells, there was no statistical variation as compared with control (Fig. 1C).

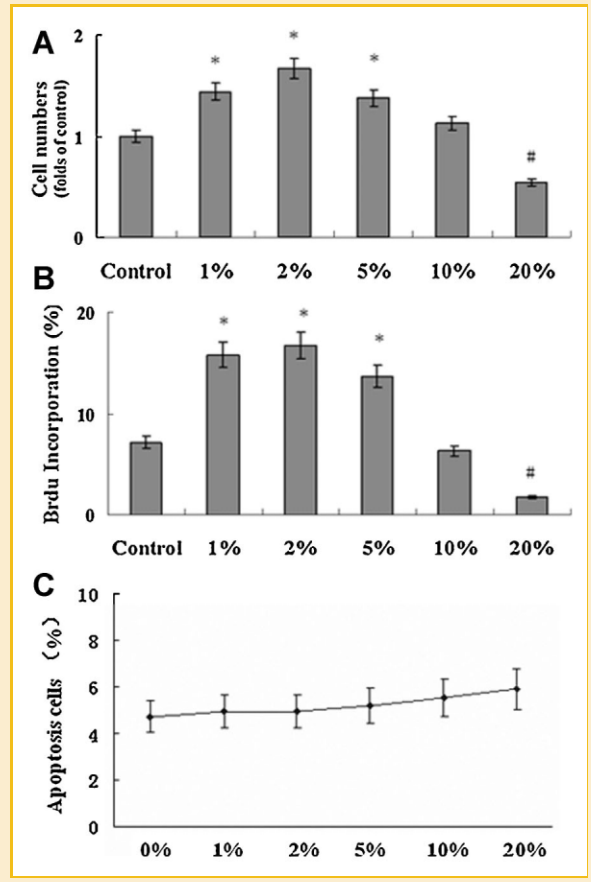

Fig. 1. Effects of CSE on rat PASMCs proliferation and apoptosis. Rat PASMCs were seeded into six-well plates and incubated with CSE at different concentrations (1–20%) for 48 h (n = 6). A: The total cells number was counted using a hemocytometer. B: The BrdU incorporation was expressed as the percentage of total cell. C: Cells apoptosis was detected by TUNEL. $^{*}P < 0.05$ as compared with control, $^{\#}P < 0.01$ as compared with control. All experiments were repeated as least thrice.

CYCLIN D1 IS REQUIRED FOR CSE-INDUCED RAT PASMCs PROLIFERATION

As shown in Figure 2, 2% CSE significantly improved cyclinD1 expression at mRNA level (0.41 ± 0.06 vs. 0.12 ± 0.03, $^{*}P < 0.01$) and protein level (0.55 ± 0.07 vs. 0.16 ± 0.03, $^{*}P < 0.01$) as compared with control. Moreover, CSE increased the BrdU incorporation significantly as compared with control (17.66 ± 2.87% vs. 7.54 ± 1.33%, $^{*}P < 0.05$).

However, the cyclin D1-specific siRNA successfully inhibited the expression of cyclin D1 at both mRNA level (0.08 ± 0.01 vs. 0.39 ± 0.05, $^{\#}P < 0.05$) and protein level (0.12 ± 0.01 vs. 0.58 ± 0.06, $^{\#}P < 0.05$). Furthermore, cyclin D1-specific siRNA also markedly decreased the BrdU incorporation as compared with negative control (10.26 ± 1.52% vs. 16.48 ± 2.41%, $^{\#}P < 0.05$).

PMA PROMOTED RAT PASMCs PROLIFERATION VIA CYCLIN D1 PATHWAY

Figure 3 shows the effect of PMA, a PKCα-specific activator, on cells proliferation. As compared with control, treatment with PMA (100 nM) significantly induced the cyclin D1 expression (0.19 vs. 0.83, $^{*}P < 0.01$ in Fig. 3B) and BrdU incorporation (9.6% vs. 29.3%, $^{*}P < 0.01$ in Fig. 3C). The specific siRNA not only decreased cyclin D1 protein levels (0.41 vs. 0.75, $^{\#}P < 0.05$ in Fig. 3B) but also suppressed the BrdU incorporation (19.2% vs. 27.7%, $^{\#}P < 0.05$ in Fig. 3C) as compared with negative control.

PKCα-SPECIFIC siRNA INHIBITED CYCLIN D1 PROTEIN LEVELS

As shown in Figure 4, 2% CSE significantly improved the protein levels of PKCα (Fig. 4B, 0.78 ± 0.19 vs. 0.25 ± 0.04, $^{*}P < 0.01$) and cyclinD1 (Fig. 4C, 0.56 ± 0.15 vs. 0.19 ± 0.03, $^{*}P < 0.01$) as compared with control. PKCα-specific siRNA successfully suppressed the protein levels of PKCα as compared with negative control (Fig. 4B, 0.19 ± 0.04 vs. 0.76 ± 0.18, $^{\#}P < 0.01$). Furthermore, cyclin D1 protein levels was also inhibited by PKCα-specific siRNA as compared with negative control (Fig. 4C, 0.31 ± 0.05 vs. 0.58 ± 0.16, $^{\#}P < 0.05$). These were associated with that of mRNA levels as described above.

The protein levels of PKCα and cyclin D1 were also detected by immunofluorescence staining. The stimulation of 2% CSE increased PKCα expression in cytosol and nucleus (Fig. 5B) and cyclin D1

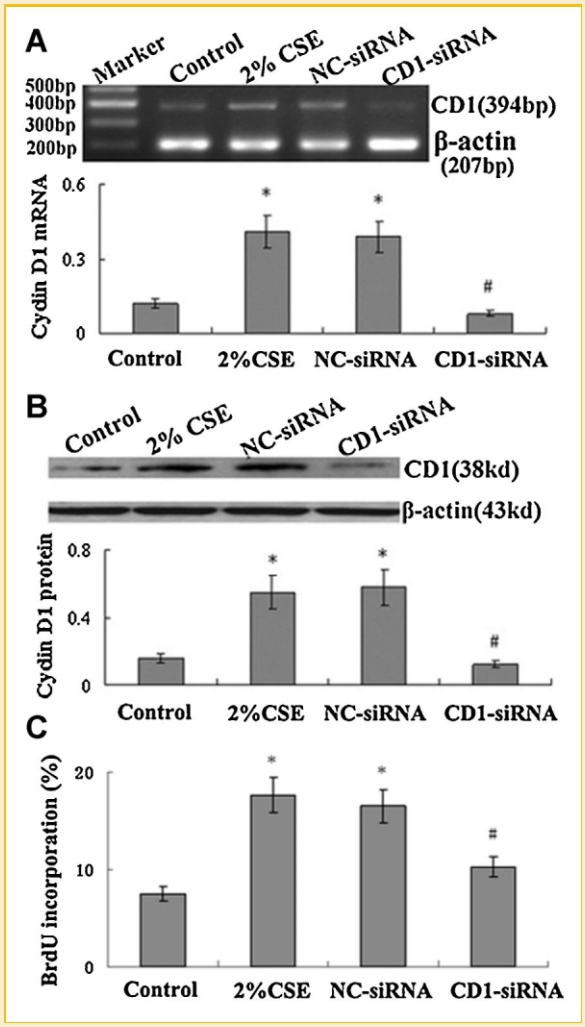

Fig. 2. Cyclin D1-specific siRNA silenced cyclin D1 expression and inhibited DNA synthesis. Rat PASMCs were transfected with cyclin D1-specific siRNA (CD1-siRNA) or negative control siRNA (NC-siRNA) and then treated with 2% CSE for 48 h (n = 6). A: The mRNA levels of cyclin D1 in different groups were evaluated by RT-PCR and expressed as the ratio of optical density of cyclin D1 and β-actin. B: The protein levels of cyclin D1 in different groups were evaluated by Western blot and expressed as the ratio of optical density of cyclin D1 and β-actin. C: DNA synthesis was measured by BrdU incorporation. $^{*}P < 0.01$ as compared with control, $^{\#}P < 0.05$ as compared with NC-siRNA. All experiments were repeated as least thrice.

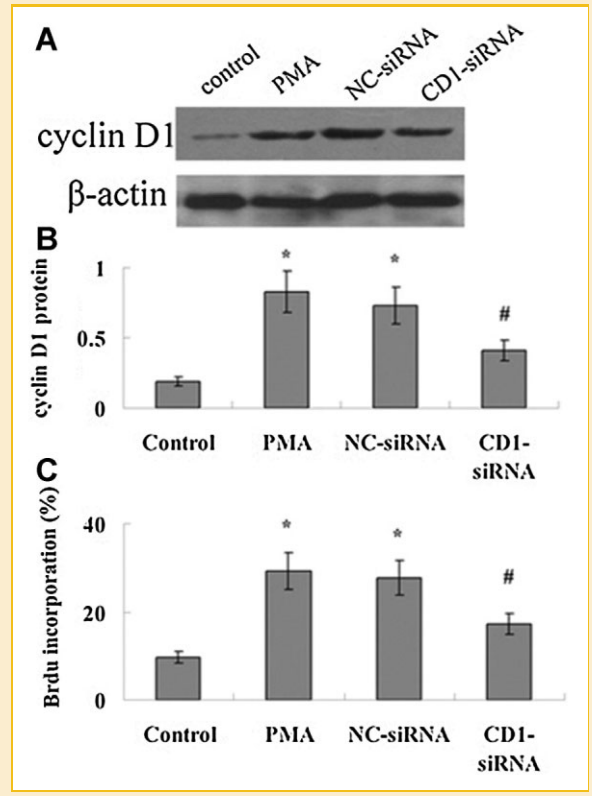

Fig. 3. PMA promoted cells proliferation via cyclin D1 pathway. Rat PASMCs were transfected with cyclin D1-specific siRNA (CD1-siRNA) or negative control siRNA (NC-siRNA) and then treated with PMA (100 nM) for 48 h (n = 6). A: Representative blots of cyclin D1 protein preformed by SDS-PAGE. B: Cyclin D1 protein levels were expressed as the optical density of cyclin D1 normalized to that of β-actin. C: DNA synthesis was measured by BrdU incorporation. $^{*}P < 0.01$ as compared with control, $^{\#}P < 0.05$ as compared with NC-siRNA. All experiments were repeated as least thrice.

expression in nucleus (Fig. 5G) as compared with control (Fig. 5A,F). But PKCα-specific siRNA not only prevented PKCα expression (Fig. 5D) but also decreased cyclin D1 protein (Fig. 5I) successfully as compared with negative control (Fig. 5C,H).

PKCα-SPECIFIC siRNA SUPPRESSED CELL-CYCLE PROGRESSION AND CELLS PROLIFERATION

Finally, we investigate the effect of PKCα-specific siRNA on cell-cycle progression and cell proliferation. As compared with control, 2% CSE significantly decreased cells percentage in G0/G1 phase (Fig. 6A, $62.19 \pm 8.64\%$ vs. $85.95 \pm 12.37\%$, $^{*}P < 0.01$) and increased cells percentage in S phase (Fig. 6A, $18.36 \pm 4.13\%$ vs. $8.48 \pm 1.24\%$, $^{*}P < 0.01$). However, PKCα-specific siRNA significantly increased the percentage of cells in S phase ($13.23 \pm 2.27\%$ vs. 17.49 ± 2.64, $^{\#}P < 0.05$) and caused a G0/G1 cell-cycle arrest ($75.29 \pm 15.35\%$ vs. $63.16 \pm 16.22\%$, $^{\#}P < 0.05$) as compared with negative control.

Figure 6B shows the results of cells count, which were similar to that of cell-cycle analysis. PKCα-specific siRNA decreased the cells number of CSE-treated rat PASMCs by about 29.88% ($^{\#}P < 0.05$ as compared with negative control). Although PKCα-specific siRNA successfully cause G0/G1 cell-cycle arrest in vitro, it did not completely blocked the cells proliferation as compared with control (Fig. 6C, 1.37-folds vs. 1-fold, $^{+}P < 0.05$).

DISCUSSION

Cigarette smoke might induce DNA synthesis and cells proliferation in many kinds of mammalian cells, including SMCs from vessels and airways [Nishio and Watanabe, 1998; Pera et al., 2010]. In this study, there is a biphasic nature of the effect of CSE on cells proliferation. CSE at a low concentration promoted cells proliferation, but CSE at a high concentration inhibited cells growth. Cigarette smoke contains

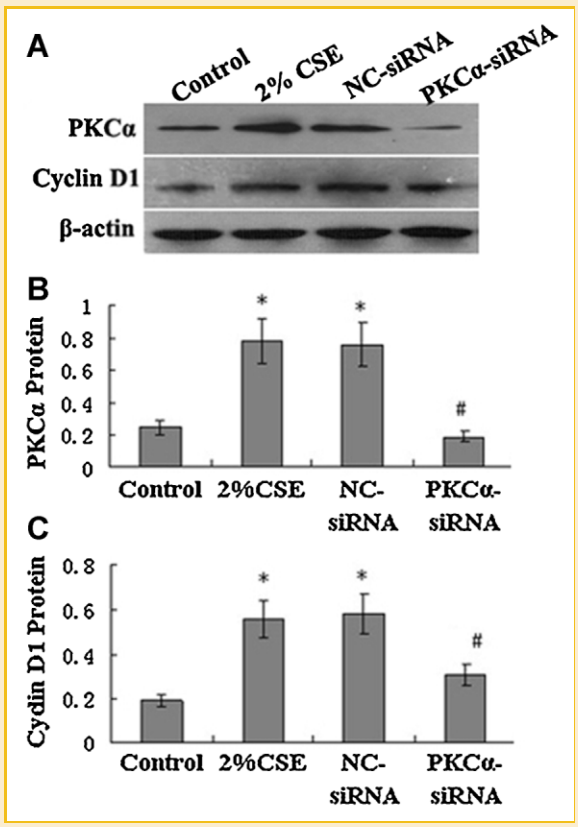

Fig. 4. PKCα-specific siRNA suppressed the protein levels of PKCα and cyclin D1. Rat PASMCs were transfected with PKCα-specific siRNA (PKCα-siRNA) or negative control siRNA (NCsiRNA), and then treated with 2% CSE for 48 h ($n = 6$). A: Representative blots of PKCα and cyclin D1 protein preformed by SDS–PAGE. B: PKCα protein levels were expressed as the optical density of PKCα normalized to that of β-actin. C: Cyclin D1 protein levels were expressed as the optical density of PKCα normalized to that of β-actin. $^{*}P < 0.01$ as compared with control, $^{\#}P < 0.05$ as compared with NC-siRNA. All experiments were repeated as least thrice.

a great number of distinct chemical compounds. Some compounds (such as nicotine and cotinine) are mitogenic for vascular SMCs at low doses [Carty et al., 1997; Li et al., 2004], while high doses of these compounds had a toxic effect on cells proliferation [Jacob et al., 2009]. Some other compounds (such as carbon monoxide and nitric oxide) might directly inhibit the proliferation of vascular SMCs [Cornwell et al., 1994; Morita et al., 1997]. Furthermore, previous study has reported that CSE enhanced SMCs proliferation through the dysregulation of the activities of superoxide dismutase and glutathione [Nishio and Watanabe, 1998]. As the concentration of CSE varied, cells growth was affected by the relative quantities of all these compounds. So, the affect of these compounds on the activation of PKCα and cyclin D1 need to be further investigated. However, the high concentrations of CSE are actually to be less relevant in vivo [Su et al., 1998]. For example, the concentration of cotinine in a passive smoker is significant lower than the concentration which was cytotoxic to vascular SMCs [Carty et al., 1997; Ambalavanan et al., 2001; Jacob et al., 2009].

Cyclin D1 is one of the most important regulators of G1 cell-cycle progression. As a critical compound in cigarette smoke, nicotine could activate cyclin D1 in epithelial cells, preosteoblastic cells, and vascular SMCs [Chu et al., 2005; Pestana et al., 2005; Sato et al., 2008]. In this study, CSE-activated cyclin D1 expression and then accelerated cells proliferation. These results were agreed with previous studies on SMCs from intestine, airways and vessels [Kuemmerle et al., 2004; Pera et al., 2010; Zeng et al., 2010]. When associated with cyclin-dependent kinase 4/6, cyclin D1 could trigger major mitotic events by phosphorylating certain target proteins on chromosomes or elsewhere [Gladden and Diehl, 2005].

As an important intracellular mediator in signal transduction, PKCα could also be activated by nicotine in many kinds of cells, such as lung epithelial cells, cancer cells, and vascular endothelial cells [Yang et al., 2006; Guo et al., 2008; Nishioka et al., 2010]. As a specific activator to PKCα, PMA could induce cells proliferation in our study, which was similar to that of CSE. Moreover, PKCα was also activated by CSE in rat PASMCs. Nicotine might also participate

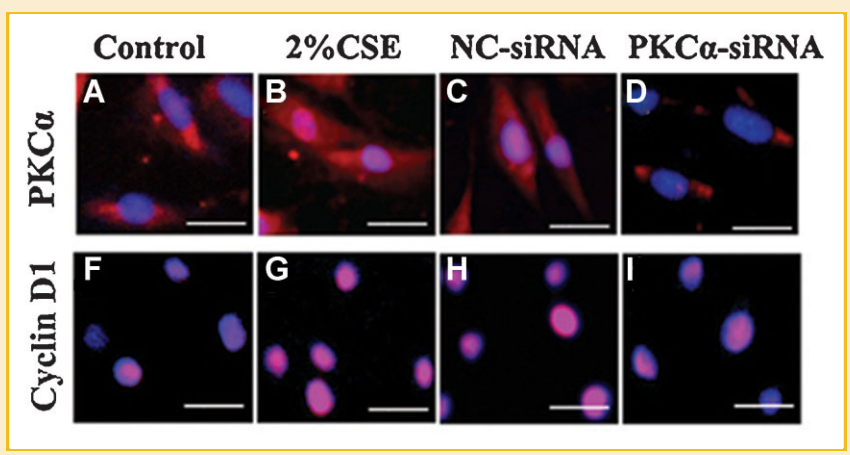

Fig. 5. PKCα-specific siRNA inhibited the protein expression of PKCα and cyclin D1. Rat PASMCs were transfected with PKCα-specific siRNA (PKCα-siRNA) or negative control siRNA (NCsiRNA) and then treated with 2% CSE for 48 h ($n = 6$). Cells in groups of control (A,F), 2%CSE (B,G), NC-siRNA (C,H), and PKCα-siRNA (D,I) were incubated with mouse anti-PKCα or anti-cyclin D1 antibodies, respectively, and then stained by Cy3-labeled goat anti-mouse IgG (red) and DAPI (blue). Magnification: 400×. Scale bars: 100 μm.

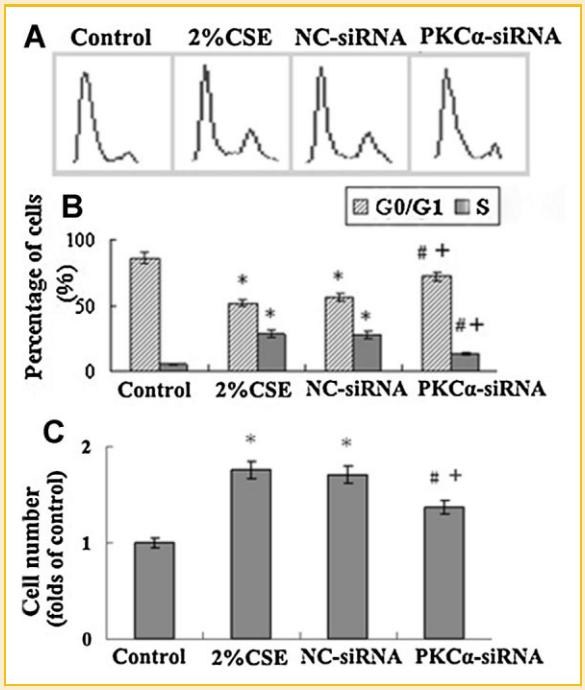

Fig. 6. PKCα-specific siRNA caused cell-cycle arrest at G0/G1 phase and inhibited cells proliferation. Rat PASMCs were transfected with PKCα-specific siRNA (PKCα-siRNA) or negative control siRNA (NCsiRNA) and then treated with 2% CSE for 48 h ($n = 6$). A: Cell-cycle analysis was evaluated by flow cytometry. B: The proliferation of rat PASMCs was measured by cells count. $^{*}P < 0.01$ as compared with control, $^{\#}P < 0.05$ as compared with NC-siRNA, $^{+}P < 0.05$ as compared with control. All experiments were repeated as least thrice.

in this process. Although the specific siRNA successfully inhibited PKCα activation, it did not prevent cells proliferation completely. These might due to two reasons. Firstly, other PKC isoforms (such as PKC beta, delta or epsilon) or some other mediators (such as extracellular signal-regulated kinase or signal transducer and activator of transcription) might also be involved in this process [Itoh et al., 2001; Smani et al., 2008]. Secondly, specific siRNA did not always silence the targeted gene completely because of the differences of target sequences and knockdown efficiency [Tiemann and Rossi, 2009]. In our study, the specific siRNA blocked PKCα expression about 76%. As the roles of other PKC isoforms in rat PASMCs proliferation were undetermined, we did not use any PKC inhibitors as a positive control.

Increasing evidences have demonstrated PKC as a key regulator of cell-cycle transitions, including the G1/S and G2/M checkpoints [Black, 2000]. As one of the most important regulators in G1 cell cycle, cyclin D1 has been proved to be a target of PKC in many kinds of cells, such as epithelial cells, renal proximal tubule cells and SMCs [Kampfer et al., 2001; Page et al., 2002; Bowles et al., 2007; Lee and Han, 2008]. This study revealed that PKCα promoted cell-cycle progression and cells proliferation by activating cyclin D1 expression. This was contrast with other studies which implied that PKCα suppressed cyclin D1 expression in intestinal epithelium and prostate cancer cells [Hizli et al., 2006; Paone et al., 2008]. These might due to the different cell lines or the different stimuli for PKCα activation. On the one hand, the same isozyme of PKC might play contrast roles in different cells types. For example, PKCα activation promoted the proliferation of airway SMCs, but suppressed the proliferation of aortas SMCs [Haller et al., 1995; Qiao et al., 2008]. On the other hand, activation of PKC by different stimulus resulted in its translocation to distinct cellular compartments, such as plasma membrane or nucleus [Nakashima 2002; Alzamora and Harvey, 2008]. The differential translocation of PKC might lead to different downstream targets and result in different functions [Kiley and Parker, 1995; Konopatskaya and Poole, 2010]. In our study, CSE might activate PKCα and then induce its translocation to nucleus. The translocation of PKCα to nucleus might result in the activation of cyclin D1 and the cell-cycle progression. Previous studies have demondtrated that PKC regulated cyclin D1 transcription and translation via different ways [Page et al., 2002; Hizli et al., 2006]. However, it is not clear that how the PKCα translocation regulated cyclin D1 expression in CSE-treated rat PASMCs.

In this study, PKCα siRNA blocked cyclin D1 expression and subsequently inhibited cell-cycle progression and cells proliferation in rat PASMCs. These data demonstrated that PKCα-mediated cyclin D1 expression was involved in CSE-induced rat PASMCs proliferation in vitro. However, further researches are needed for a full understanding of PKCα pathway in the regulation of cyclin D1 expression and cell-cycle progression.

REFERENCES

Alzamora R, Harvey BJ. 2008. Direct binding and activation of protein kinase C isoforms by steroid hormones. Steroids 73:885–888.

Ambalavanan N, Carlo WF, Bulger A, Shi J, Philips JB III. 2001. Effect of cigarette smoke extract on neonatal porcine vascular smooth muscle cells. Toxicol Appl Pharmacol 170:130–136.

Black JD. 2000. Protein kinase C-mediated regulation of the cell cycle. Front Biosci 5:D406–D423.

Bowles DK, Maddali KK, Dhulipala VC, Korzick DH. 2007. PKC delta mediates anti-proliferative, pro-apoptic effects of testosterone on coronary smooth muscle. Am J Physiol Cell Physiol 293:C805–C813.

Carty CS, Huribal M, Marsan BU, Ricotta JJ, Dryjski M. 1997. Nicotine and its metabolite cotinine are mitogenic for human vascular smooth muscle cells. J Vasc Surg 25:682–688.

Chu M, Guo J, Chen CY. 2005. Long-term exposure to nicotine, via ras pathway, induces cyclin D1 to stimulate G1 cell cycle transition. J Biol Chem 280:6369–6379.

Cornwell TL, Arnold E, Boerth NJ, Lincoln TM. 1994. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP dependent protein kinase by cGMP. Am J Physiol Cell Physiol 267:C1405–C1413.

Du CL, Xu YJ, Liu XS, Xie JG, Xie M, Zhang ZX, Zhang J, Qiao LF. 2010. Up-regulation of cyclin D1 expression in asthma serum-sensitized human airway smooth muscle promotes proliferation via protein kinase C alpha. Exp Lung Res 36:201–210.

Garg R, Ramchandani AG, Maru GB. 2008. Curcumin decreases 12-O-tetradecanoylphorbol-13-acetate-induced protein kinase C translocation to modulate downstream targets in mouse skin. Carcinogenesis 29:1249–1257.

Gladden AB, Diehl JA. 2005. Location, location, location: The role of cyclin D1 nuclear localization in cancer. J Cell Biochem 96:906–913.

Gould CM, Newton AC. 2008. The life and death of protein kinase C. Curr Drug Targets 9:614–625.

Guo J, Ibaragi S, Zhu T, Luo LY, Hu GF, Huppi PS, Chen CY. 2008. Nicotine promotes mammary tumor migration via a signaling cascade involving protein kinase C and CDC42. Cancer Res 68:8473–8481.

Gwak J, Jung SJ, Kang DI, Kim EY, Kim DE, Chung YH, Shin JG, Oh S. 2009. Stimulation of protein kinase C-alpha suppresses colon cancer cell proliferation by down-regulation of beta-catenin. J Cell Mol Med 13:2171–2180.

Haller H, Lindschau C, Quass P, Distler A, Luft FC. 1995. Differentiation of vascular smooth muscle cells and the regulation of protein kinase C-α. Circ Res 76:21–29.

Hizli AA, Black AR, Pysz MA, Black JD. 2006. Protein kinase C α signaling inhibits cyclin D1 translation in intestinal epithelial cells. J Biol Chem 281:14596–14603.

Hu J, Xu YJ, Zhang ZX, Tian F. 2007. Effect of cigarette smoke extract on proliferation of rat pulmonary artery smooth muscle cells and the relevant roles of protein kinase C. Chin Med J 120:1523–1528.

Itoh H, Yamamura S, Ware JA, Zhuang S, Mii S, Liu B, Kent KC. 2001. Differential effects of protein kinase C on human vascular smooth muscle cell proliferation and migration. Am J Physiol Heart Circ Physiol 281:H359–H370.

Jacob T, Clouden N, Hingorani A, Ascher E. 2009. The effect of cotinine on telomerase activity in human vascular smooth muscle cells. J Cardiovasc Surg 50:345–349.

Kamper S, Windegger M, Hochholdinger F, Schwaiger W, Pestell RG, Baier G, Grunicke HH, Uberall F. 2001. Protein kinase C isoforms involved in the transcriptional activaction of cyclin D1 by transforming Ha-Ras. J Bio Chem 276:42834–42842.

Kiley SC, Parker PJ. 1995. Differential localization of protein kinase C isozymes in U937cells: Evidence for distinct isozyme functions during monocyte differentiation. J Cell Sci 108:1003–1016.

Konopatskaya O, Poole AW. 2010. Protein kinase C alpha: Disease regulator and therapeutic target. Trends Pharmacol Sci 31:8–14.

Kuemmerle JF, Zhou H, Bowers JG. 2004. IGF-I stimulates human intestinal smooth muscle cell growth by regulation of G1 phase cell cycle proteins. Am J Physiol Gastrointest Liver Physiol 286:G412–G419.

Kundumani-Sridharan V, Wang D, Karpurapu M, Liu Z, Zhang C, Dronadula N, Rao GN. 2007. Suppression of activation of signal transducer and activator of transcription-5B signaling in the vessel wall reduces balloon injury-induced neointima formation. Am J Pathol 171:1381–1394.

Lee YJ, Han HJ. 2008. Albumin-stimulated DNA synthesis is mediated by Ca^{2+}/PKC as well as EGF receptor-dependent p44/42 MAPK and NF-kB signal pathways in renal proximal tubule cells. Am J Physiol Renal Physiol 294:F534–F541.

Li JM, Cui TX, Shiuchi T, Liu HW, Min LJ, Okumura M, Jinno T, Wu L, Iwai M, Horiuchi M. 2004. Nicotine enhances angiotensin II-induced mitogenic response in vascular smooth muscle cells and fibroblasts. Arterioscler Thromb Vasc Biol 24:80–84.

Marino M, Acconcia F, Bresciani F, Weisz A, Trentalance A. 2002. Distinct nongenomic signal transduction pathways controlled by 17beta-estradiol regulate DNA synthesis and cyclin D1 gene transcription in HepG2 cells. Mol Biol Cell 13:3720–3729.

Morita T, Mitsialis SA, Koike H, Liu Y, Kourembanas S. 1997. Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J Biol Chem 272:32804–32809.

Nakashima S. 2002. Protein kinase C alpha (PKC alpha): Regulation and biological function. J Biochem 132:669–675.

Nishio E, Watanabe Y. 1998. Cigarette smoke extract is a modulator of mitogenic action in vascular smooth muscle cells. Life Sci 62:1339–1347.

Nishioka T, Guo J, Yamamoto D, Chen L, Huppi P, Chen CY. 2010. Nicotine, through upregulating pro-survival signaling, cooperates with NNK to promote transformation. J Cell Biochem 109:152–161.

Okazaki J, Mawatari K, Liu B, Kent KC. 2000. The effect of protein kinase C and its alpha subtype on human vascular smooth muscle cell proliferation, migration and fibronectin production. Surgery 128:192–197.

Page K, Li J, Corbit KC, Rumilla KM, Soh JW, Weinstein IB, Albanese C, Pestell RG, Rosner MR, Hershenson MB. 2002. Regulation of airway smooth muscle CyclinD1 transcription by protein kinase C-delta. Am J Respir Cell Mol Biol 27:204–213.

Paone A, Starace D, Galli R, Padula F, Cesaris PD, Filippini A, Ziparo E, Riccioli A. 2008. Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-α-dependent mechanism. Carcinogenesis 29:1334–1342.

Pera T, Gosens R, Lesterhuis AH, Sami R, Toorn M, Zaagsma J, Meurs H. 2010. Cigarette smoke and lipopolysaccharide induce a proliferative airway smooth muscle phenotype. Respir Res 11:48.

Pestana IA, Vazquez-Padron RI, Aitouche A, Pham SM. 2005. Nicotinic and PDGF-receptor function are essential for nicotine-stimulated mitogenesis in human vascular smooth muscle cells. J Cell Biochem 96:986–995.

Qiao LF, Xu YJ, Liu XS, Xie JG, Wang J, Du CL, Zhang J, Ni W, Chen SX. 2008. PKC promotes proliferation of airway smooth muscle cells by regulating cyclinD1 expression in asthmatic rats. Acta Pharmacol Sin 29:677–686.

Rudkouskaya A, Chernoguz A, Haskew-Layton RE, Mongin AA. 2008. Two conventional protein kinase C isoforms, α and betaI, are involved in the ATP-induced activation of volume-regulated anion channel and glutamate release in cultured astrocytes. J Neurochem 105:2260–2270.

Sato T, Abe T, Nakamoto N, Tomaru Y, Koshikiya N, Nojima J, Kokabu S, Sakata Y, Kobayashi A, Yoda T. 2008. Nicotine induces cell proliferation in association with cyclin D1 up-regulation and inhibits cell differentiation in association with p53 regulation in a murine pre-osteoblastic cell line. Biochem Biophys Res Commun 377:126–130.

Smani T, Patel T, Bolotina VM. 2008. Complex regulation of store-operated Ca^{2+} entry pathway by PKC-epsilon in vascular SMCs. Am J Physiol Cell Physiol 294:C1499–C1508.

Stacey DW. 2003. Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Curr Opin Cell Biol 15:158–163.

Su Y, Han W, Giraldo C, De Li Y, Block ER. 1998. Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. Am J Respir Cell Mol Biol 19:819–825.

Tiemann K, Rossi JJ. 2009. RNAi-based therapeutics-current status, challenges and prospects. EMBO Mol Med 1:142–151.

Wright JL, Levy RD, Churg A. 2005. Pulmonary hypertension in chronic obstructive pulmonary disease: Current theories of pathogenesis and their implications for treatment. Thorax 60:605–609.

Wu TT, Hsieh YH, Hsieh YS, Liu JY. 2008. Reduction of PKC α decreases cell proliferation, migration, and invasion of human malignant hepatocellular carcinoma. J Cell Biochem 103:9–20.

Yang T, Roder KE, Bhat GJ, Thekkumkara TJ, Abbruscato TJ. 2006. Protein kinase C family members as a target for regulation of blood-brain barrier Na,K,2Cl-cotransporter during in vitro stroke conditions and nicotine exposure. Pharm Res 2:291–302.

Zeng DX, Liu XS, Xu YJ, Wang R, Xiang M, Xiong WN, Ni W, Chen SX. 2010. Plasmid-based short hairpin RNA against cyclin D1 attenuated pulmonary vascular remodeling in smoking rats. Microvasc Res 80:116–122.