Nicotine enhances store-operated calcium entry by upregulating HIF-1α and SOCC components in non-small cell lung cancer cells

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Abstract. Store-operated calcium entry (SOCE) is critical for regulating the proliferation and metastasis of various cancer types. The present study aimed to investigate the role of SOCE on nicotine-promoted proliferation of non-small cell lung cancer (NSCLC) A549 cells. Cell proliferation was evaluated by BrdU incorporation assay. The SOCE and basal $[Ca^{2+}]_i$ in NSCLC A549 cells were determined using Fura-2 fluorescence microscopy. The mRNA and protein expression levels were determined by real-time quantitative PCR and western blotting, respectively. The results demonstrated that, in A549 cells, the detectable store-operated calcium channel (SOCC) components were TRPC proteins 1, 3, 4 and 6 and Orail, among which TRPC1, TRPC6 and Orai1 are expressed at relatively high levels with TRPC3 and TRPC4 at relatively low levels. Nicotine upregulated the mRNA and protein expression of TRPC1, TRPC6 and Orai1, increased basal $[Ca^{2+}]_i$ and enhanced SOCE. Promotion of cell proliferation but not migration was observed in the nicotine-treated cells, which was inhibited by SOCE inhibitor SKF-96365. Furthermore, nicotine upregulated HIF-1α expression in the A549 and NCI-H292 cells. Silencing of HIF-1α abrogated the increases in TRPCs and Orail and reversed the increases in basal $[Ca^{2+}]_i$ and SOCE. Meanwhile, suppression of proliferation was observed in cells following HIF-1α silencing. In conclusion, the results indicate that nicotine promotes lung cancer cell proliferation likely by upregulating HIF-1α and SOCC components and therefore enhancing SOCE and increasing basal $[Ca^{2+}]_i$.

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Introduction

Lung cancer is the most commonly diagnosed cancer and the most common cause of cancer-related death worldwide (1). Approximately 85% of lung cancer cases are attributed to smoking, not including those occurring in nonsmokers exposed to second-hand smoke (2). As a major component in cigarette smoke, nicotine contributes to tumor progression by activating angiogenesis, promoting cell proliferation and invasion, and inhibiting apoptosis, although it does not provoke tumorigenesis (3,4). Calcium-mediated signal transduction is suggested to be one of the underlying mechanisms involved in the tumor-promoting effects induced by nicotine/nicotinic receptors (5,6). In tumor progression, the diversity of calcium channels, mostly non-voltage gated calcium channels, on the plasma membrane is relevant to the differential behaviors in proliferation and migration of cancer cells (7). Store-operated Ca^{2+} entry (SOCE), activated by intracellular Ca^{2+} store depletion, is the primary mechanism for Ca^{2+} influx in non-exitable cells (8). It has been found that SOCE remodeling is associated with tumor progression in various human cancers (9,10).

The Orai channels and Ca^{2+}-permeable transient receptor potential canonical (TRPC) channels are Ca^{2+}-permeable channels involved in SOCE upon the binding of the stromal interaction molecule (STIM) proteins as Ca^{2+} sensors (11). There are three mammalian Orai homologues (Orai1-3) showing differences in response to the process of Ca^{2+} depotentiation (12) and six TRPC isoforms (TRPC1-6) serving as non-selective Ca^{2+}-permeable cation channels, through which the Ca^{2+} current in SOCE is generated by the formations of Orai1-STIM1 or TRPCs-STIM1 components (13,14).

Hypoxia is a common feature of solid tumor masses and is essential for the formation of the cellular and physiologic characteristics of cancer (15). The association between hypoxia and intracellular $[Ca^{2+}]_i$ regulation has been identified in hepatoma cells, in which HIF-1α, a key regulator for the adaption of cancer cells to a low-oxygen microenvironment, enhanced STIM1 transcription and contributed to SOCE

and tumorigenesis (16,17). Furthermore, both inhibition of TRPC6-mediated calcium signaling and attenuation of HIF-1α signaling elevated the sensitivity of tumor cells to drugs (18).

In human non-small cell lung cancer cells, it has been found that nicotine induced HIF-1α overexpression (19). Excessive HIF-1α expression was associated with the growth of lung cancer A549 cells *in vitro* and *in vivo* (20). Nevertheless, the association between nicotine-induced HIF-1α change and SOCE in lung tumor cell growth remains unclear. In this present study, we evaluated the effects of nicotine on changes in the expression of store-operated calcium channel (SOCC) components and SOCE in A549 cells. We demonstrated overexpression of HIF-1α-mediated SOCC components enhancement of SOCE in the presence of nicotine.

Materials and methods

Reagents. (-)-Nicotine ditartrate was purchased from Calbiochem (San Diego, CA, USA; cat. no. 481975). Nifedipine, cyclopiazonic acid (CPA) and SKF-96365 were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Fluorescent dye fura-2 AM was from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture and treatment. The non-small cell lung cancer cell line A549 was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. For nicotine treatment, the cells were seeded into 6-well plates at a density of $1x10^5$ cells per well, grown until 70% confluence and exchanged with serum-free medium for a 12-h culture to reach growth arrest. Then, the cells were treated with nicotine in medium containing 0.1% FBS for the indicated time before being collected for further analyses.

RNA extraction and quantitative real-time PCR. Total RNA in cultured cells was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse transcription into cDNA was performed from 1 μg total RNA and quantitative real-time PCR was carried out by using Scofast™ EvaGreen superMix (Bio-Rad Laboratories, Hercules, CA, USA) and primers listed in Table I. The relative concentration of each transcript was calculated using the Pfaffl method (21) and normalized to 18S as an internal control for each sample.

RNA interference. For the transient silencing of HIF-1α gene expression, small interfering RNA (siRNA) targeting to HIF-1α (siHIF-1α, 5'-CCACCACUGAUGAAUUAAATT-3') and negative control small interfering RNA (siNT, 5'-UUCUCC GAACGUGUCACGUTT-3') were transfected into A549 cells using HiPerFect Transfection Reagent (Qiagen, Duesseldorf, Germany) at a final concentration of 5 nM for 24 h. Then the cells were subjected to nicotine treatment for 24 or 48 h.

Western blotting. Cells were homogenized in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EGTA, 2 mM EDTA, 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich Inc.). Equal amount of total proteins for each sample was separated on SDS-PAGE gel, blotted with primary antibodies against human TRPC1 (1:1,000; rabbit polyclonal; cat. no. ACC-010; Alomone Labs, Jerusalem, Israel), TRPC6 (1:1,000; rabbit polyclonal; cat. no. ACC-017; Alomone Labs), Orai1 (1:1,000; rabbit polyclonal; cat. no. 4281; ProSci, Inc., San Diego, CA, USA), HIF-1α (1:1,000; mouse monoclone; cat. no. ab113642; Abcam, Cambridge, UK) or β-actin (1:1,000; mouse monoclone; cat. no. ab8226; Abcam) and then with the corresponding HRP-conjugated secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Finally, the signals were visualized with enhanced chemiluminescence reagents (ECL; Bio-Rad Laboratories).

Table I. Primers used for quantitative RT-PCR.

TRPC1	Forward:	5' TTGTGGAGGTGGAATTCAGG 3'
	Reverse:	5' CGTTTGTCA AGAGGCTCGTC 3'
TRPC2	Forward:	5' TCATGGTCATTGTGCTGCTC 3'
	Reverse:	5' ACTCCACGTCAGCATCATCC 3'
TRPC3	Forward:	5' CAGCCAACACGTTATCAGCA 3'
	Reverse:	5' CCTCAGTTGCTTGGCTCTTG 3'
TRPC4	Forward:	5' CGAAAGGGTTAACCTGCAAA 3'
	Reverse:	5' CAGGGACTGCAGTGTCTCAA 3'
TRPC5	Forward:	5' GTGCTGCTGAACATGCTGAT 3'
	Reverse:	5' GCTTCGTCCTTGCAAACTTC 3'
TRPC6	Forward:	5' CAGACAATGGCGGTCAAGTT 3'
	Reverse:	5' TGGTCCACGCATTATCTTCC 3'
TRPC7	Forward:	5' GTTAAAACCCTGCCAAACGA 3'
	Reverse:	5' TCCCAGATTTCCTTGCATTC 3'
HIF-1α	Forward:	5' TGCTTGGTGCTGATTTGTGAACC 3'
	Reverse:	5' CTGTCCTGTGGTGACTTGTCC 3'
Orai1	Forward:	5' ACGTGCACAATCTCAACTCG 3'
	Reverse:	5' AGCACCACCTCAGCTAGGAA 3'
18S	Forward:	5' GCAATTATTCCCCATGAACG 3'
	Reverse:	5' GGCCTCACTAAACCATCCAA 3'

Proliferation analysis. Cell proliferation was evaluated by using a colorimetric BrdU cell proliferation assay kit (Roche, South San Francisco, CA, USA) according to the manufacturer's instructions. Cell proliferation was quantified by BrdU incorporation and expressed as a multiple of the value of the control cells.

Scrape-wound migration assay. A scrape-wound migration assay was used to assess the effects of nicotine on cell mobility. A wound was produced in a confluent monolayer of A549 cells by scraping the cells with a pipette tip. Then, the cells were replenished with DMEM containing 0.1% FBS with nicotine (1 μM) and/or SKF-96365 (1 μM) to drive cell migration. Bright-field images of the wound area were captured at 0 and

24 h post-wounding with a Leica (DMI3000B) microscope (Leica Microsystems, Frankfurt, Germany) and the total number of pixels in the empty spaces inside the wound were counted using Adobe Photoshop CS5. At least three photographs were taken per group at each time-point. The migration capacity was calculated as the empty space at 0 h minus the empty space at 24 h and was represented as a percentage relative to the control.

Measurement of intracellular $[Ca^{2+}]_i$ and SOCE by fura-2 fluorescence. Intracellular $[Ca^{2+}]_i$ and SOCE were measured according to methods described previously (22). A549 cells seeded on coverslips were incubated with 5 μM fura-2 AM for 1 h in the dark at room temperature. Then, the coverslips were perfused with physiological salt solution (PSS, 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose) for 10 min to remove the extracellular fura-2 AM. Basal $[Ca^{2+}]_i$ was determined at 12-sec intervals from the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F340/F380) measured using a xenon lamp (Lambda DG4; Sutter Instrument Company, Novato, CA, USA) in 20 to 30 cells.

After basal $[Ca^{2+}]_i$ measurement, the cells were perfused with $[Ca^{2+}]$-free PSS containing 0.5 mM EGTA for 5 min to chelate residual Ca^{2+} and then with PSS containing 5 μM nifedipine and 10 μM CPA to prevent calcium entry through L-type VOCC and to deplete SR Ca^{2+} stores. To assess SOCE, the peak increase in $[Ca^{2+}]_i$ (ratio F340/F380) caused by restoration of extracellular Ca^{2+} (2.5 mM Ca^{2+} in PSS perfusate containing nifedipine and CPA) was determined.

Statistical analysis. All experiments were repeated three times. Data were statistically analyzed using the two-tailed Student's t-test and are represented as means ± SEM. $^{*}P<0.05$ and $^{**}P<0.01$ indicate a significant and extremely significant difference, respectively.

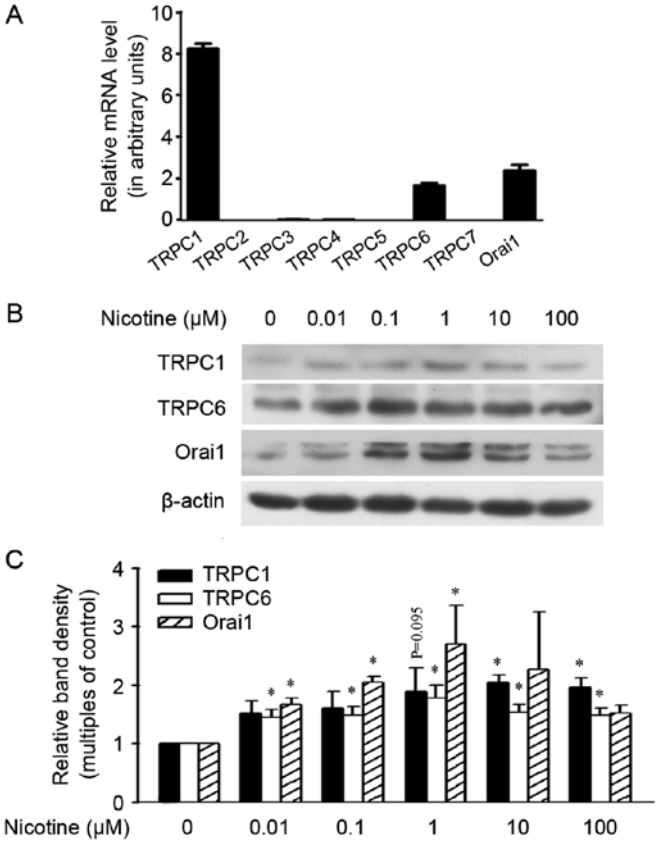

Figure 1. Nicotine induces changes in the expression of SOCC components. (A) Basal mRNA levels of store-operated calcium channel (SOCC) components, TRPC family members 1-7 and Orai1, were determined by quantitative RT-PCR in A549 cells. (B) A549 cells were exposed to nicotine (0-100 μM) for 48 h. The protein levels of TRPC1, TRPC6 and Orai1 were determined using western blotting. The results are from one representative experiment out of three similar experiments. (C) The intensity of each band was quantified, normalized to internal control β-actin and expressed as the multiple of the samples without nicotine exposure. The results are from experiments conducted in triplicate and data are presented as mean ± SEM. $^{*}P<0.05$, cells exposed to nicotine vs. cells without nicotine exposure.

Results

Nicotine upregulates the expression of SOCC components in A549 cells. The calcium channel SOCCs in mammalian cells are thought to be composed of TRPCs, Orai1 and STIM1 (23). In NSCLC A549 cells, among the seven TRPC members, TRPC1 and TRPC6 were expressed at relatively high levels with relatively low levels of TRPC3 and TRPC4. The expression levels of the other three TRPCs members, TRPC2, TRPC5 and TRPC7, were not detected in our study (Fig. 1A). The levels of TRPC6 and Orai1 in A549 cells were upregulated following exposure to nicotine at a dose as low as 0.01 μM, and were further upregulated by higher levels of nicotine in a dose-dependent manner (1-100 μM). The protein TRPC1 was not increased by nicotine at a dose lower than 1 μM, although a trend of upregulation was observed at the concentration 1 μM. Further increased nicotine dosages of 10 or 100 μM did not induce further upregulation of the proteins TRPC6 and Orai1 when compared with the dose at 1 μM (Fig. 1B and C), but did induce obvious cytotoxicity-like cell death. In human smokers, the average peak plasma nicotine level in smoking is around 10-50 mg/ml (~60-310 nM) (24). Therefore, we chose to expose cells to 1 μM nicotine in the present study.

Nicotine increases SOCE and basal intracellular $[Ca^{2+}]_i$ level. To examine the effects of nicotine on calcium influx, we measured the SOCE and basal $[Ca^{2+}]_i$ in A549 cells. A 48-h exposure to nicotine increased basal $[Ca^{2+}]_i$ (Fig. 2A and B). After washing the cells with Ca^{2+}-free PSS containing 10 μM CPA and 5 μM nifedipine, a low pulse of $[Ca^{2+}]_i$ increase was detected at 10 min which indicated store calcium release and the peak $[Ca^{2+}]_i$ at 20 min reflected SOCE resulting from the restoration of extracellular Ca^{2+} at 2.5 mM. Basically, nicotine exposure enhanced basal $[Ca^{2+}]_i$ and SOCE of A549 cells (Fig. 2A and C).

Blockage of SOCE prevents cell proliferation upon nicotine exposure. Nicotine has been reported to promote the proliferation and migration of A549 cells (25). In our study, a scrape-wound migration assay was conducted to evaluate cell migration capacity. As shown in Fig. 3A and B, nicotine (1 μM in 0.1% FBS) did not induce obvious accelerated cell migration when compared with those cells without nicotine exposure at 24 h. However, TRPC inhibitor SKF-96365 effectively abrogated cell migration, no matter whether nicotine

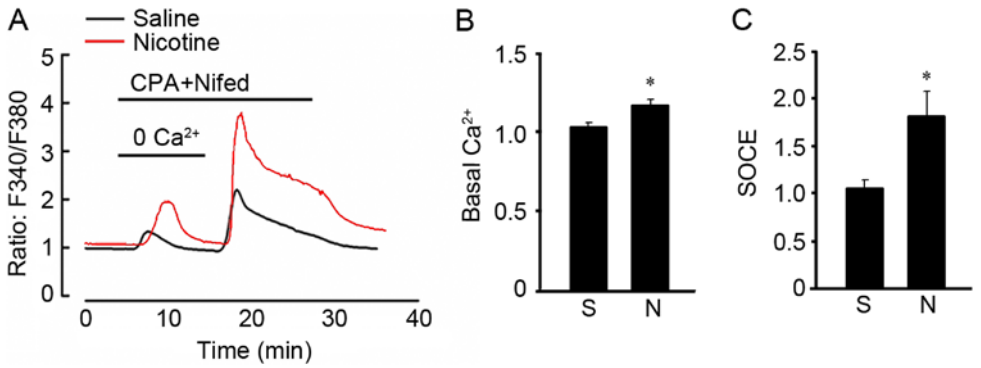

Figure 2. Effects of nicotine on basal $[Ca^{2+}]_i$ and SOCE in A549 cells. A549 cells were exposed to saline (vehicle) or nicotine (1 μM) for 48 h. After the cells were incubated with 5 μM fura-2 AM, the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F340/F380) was determined. (A) Time course of $[Ca^{2+}]_i$ before and after restoration of extracellular Ca^{2+} in cells perfused with Ca^{2+}-free physiological salt solution (PSS) containing 5 μM nifedipine and 10 μM cyclopiazonic acid (CPA) (n=4 experiments in 119 cells). (B) Average basal intracellular $[Ca^{2+}]_i$ and (C) store-operated calcium entry (SOCE) (average peak $\Delta[Ca^{2+}]_i$ shown in A, obtained from cell restored extracellular Ca^{2+}). S, saline; N, nicotine. Data are presented as mean ± SEM. *P<0.05, cells treated with saline vs. cells exposed to nicotine.

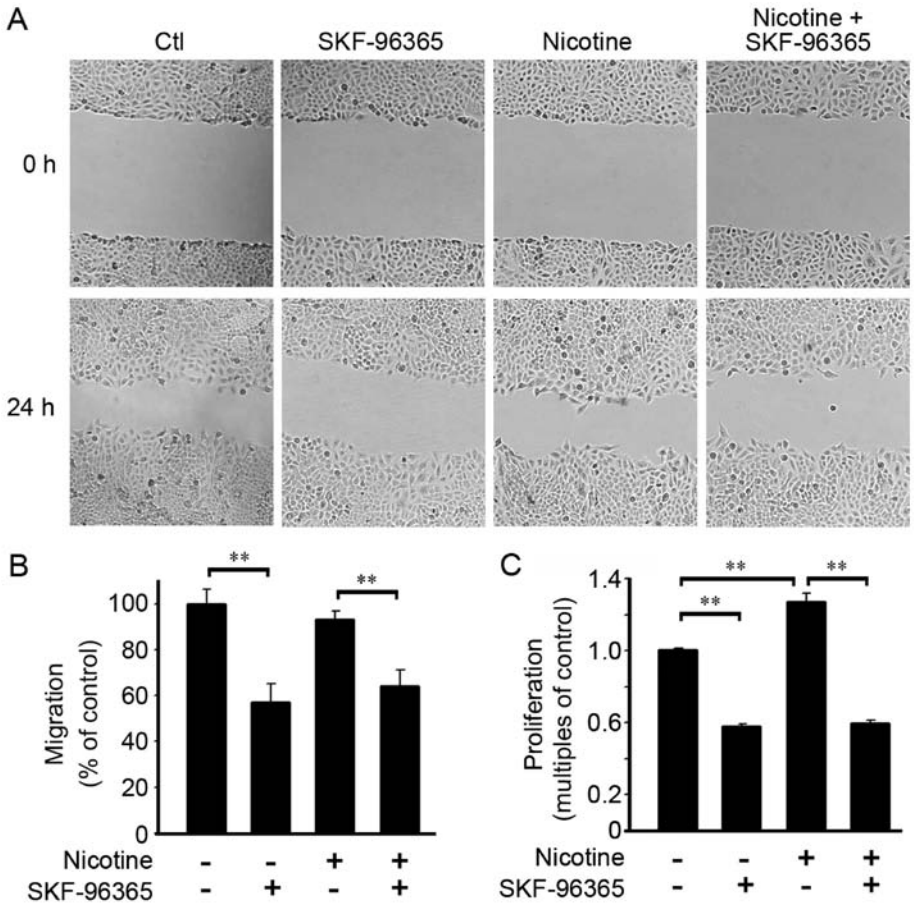

Figure 3. TRPC inhibitor SKF-96365 attenuates cell mobility and proliferation upon nicotine exposure. (A and B) A549 cells were seeded on a 6-well plate and scraped to produce a wound. Then the cells were incubated with 1 μM nicotine in the presence of TRPC inhibitor SKF-96365 (1 μM). Images of the wound area were taken at 0 and 24 h post-wounding. The cell migration is expressed as a percentage of the cells without nicotine and SKF-96365 treatments. At least three images were taken per group at each time point and the results are from one representative group of images. Results are presented as mean ± SEM. **P<0.01. (C) A549 cells were seeded on a 96-well plate and exposed to 1 μM nicotine or 1 μM SKF-96365. The cell proliferation was evaluated by BrdU incorporation assay at 48 h and is expressed as a multiple of the value of the control cells. The results are from three experiments and are presented as mean ± SEM. **P<0.01.

was present. The BrdU incorporation assay showed that nicotine enhanced cell proliferation at 48 h. The TRPC inhibitor SKF-96365 inhibited both basal and nicotine triggered cell proliferation (Fig. 3C).

HIF-1α is required for upregulation of SOCC components induced by nicotine. Since nicotine has been reported to increase HIF-1α in NSCLC cells and HIF-1α modulated expression of SOCC components in pulmonary artery smooth

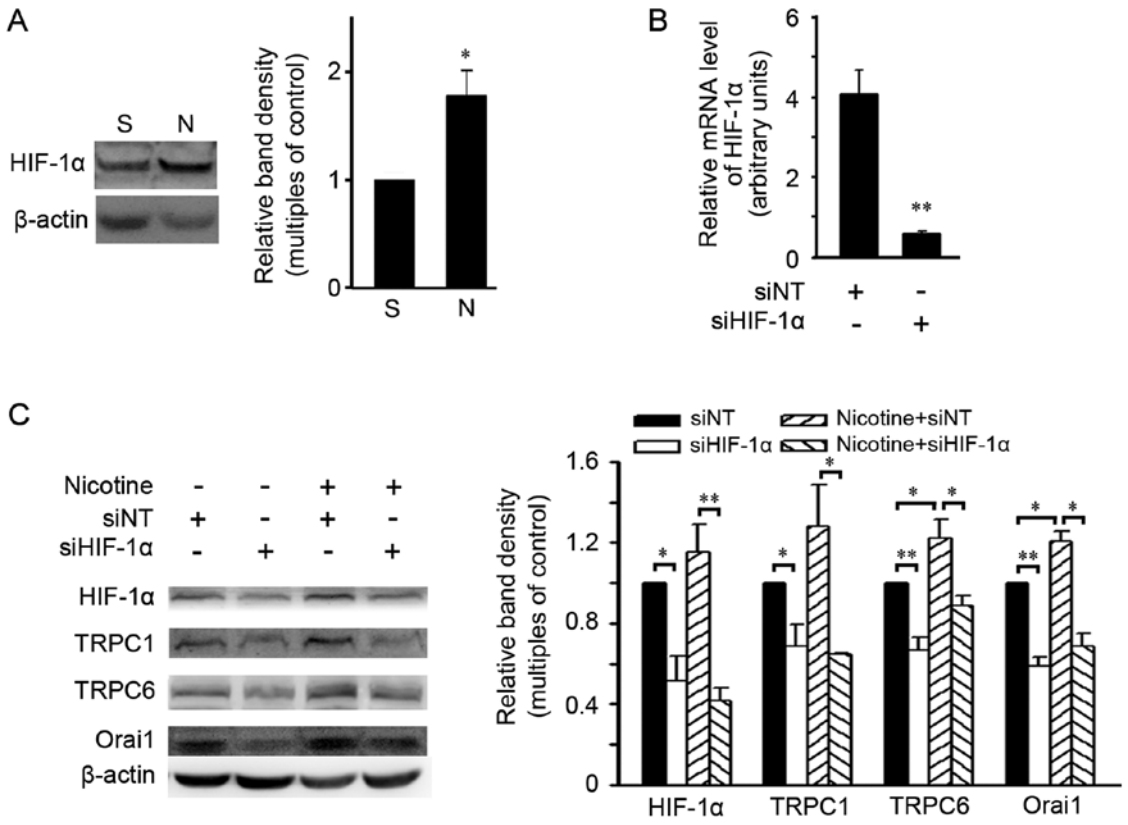

Figure 4. HIF-1α deficiency impacts expression of store-operated calcium channel (SOCC) components in A549 cells. (A) A549 cells were treated with nicotine (1 μM) for 48 h. The protein levels of HIF-1α were determined using western blotting. The relative expression of HIF-1α to β-actin is expressed as the multiple of the control cells after intensity quantification of the bands. S, saline; N, nicotine. (B) A549 cells were transfected with siHIF-1α to silence HIF-1α expression. The mRNA levels of HIF-1α were determined at 24 h after siRNA transfection. (C) Nicotine (1 μM) was used to expose cells at 24 h after siHIF-1α transfection. The protein levels of TRPC1, TRPC6 and Orai1 were determined at 48 h after nicotine exposure. The results are from one representative experiment out of three similar experiments. The intensity of the band was quantified, normalized to internal control β-actin and is expressed as the multiple of the samples treated with only siNT. Data are presented as mean ± SEM. *P<0.05 and **P<0.01.

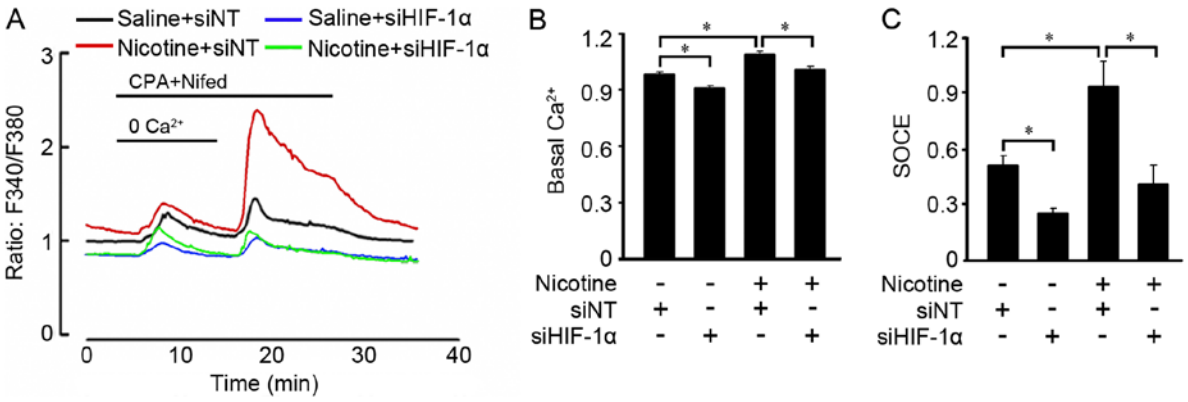

Figure 5. Basal $[Ca^{2+}]_i$ and SOCE are decreased in cells lacking HIF-1α. A549 cells underwent siHIF-1α or siNT transfection and subsequent nicotine (1 μM) exposure. After 48 h, the cells were subjected for assessment of the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F340/F380) under incubation with 5 μM fura-2 AM. (A) Time course of $[Ca^{2+}]_i$ before and after restoration of extracellular Ca^{2+} in cells perfused with Ca^{2+}-free physiological salt solution (PSS) containing 5 μM nifedipine and 10 μM cyclopiazonic acid (CPA) (n=4 experiments in 115 cells). (B) Average basal intracellular $[Ca^{2+}]_i$ and (C) store-operated calcium entry (SOCE) (average peak $\Delta[Ca^{2+}]_i$ shown in A, obtained from cells with restored extracellular Ca^{2+}). Data are presented as mean ± SEM. *P<0.05 and **P<0.01.

muscle cells (26,27), the effects of HIF-1α on nicotine-triggered expression of SOCC components were evaluated in A549 cells. As shown in Fig. 4A, nicotine induced an increase in the HIF-1α level in A549 cells at 48 h. A small interfering RNA against HIF-1α (siHIF-1α) was used to silence HIF-1α expression (Fig. 4B). After 48-h nicotine exposure, siHIF-1α reduced the basal protein levels of HIF-1α and SOCC components TRPC1, TRPC6 and Orai1, and abolished the upregulation of these proteins caused by nicotine exposure (Fig. 4C). Similar upregulatory effects of HIF-1α on SOCC components

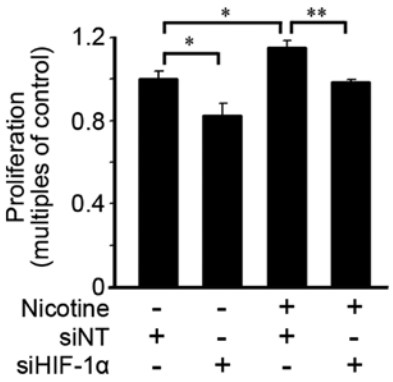

Figure 6. Cell proliferations upon siHIF-1α transfection and nicotine exposure. Cells transfected with siHIF-1α or siNT were seeded on a 96-well plate and exposed to 1 μM nicotine. The cell proliferation was evaluated by BrdU incorporation at 48 h and is expressed as the multiple of the cells transfected only with siNT. The results are from three experiments and are presented as mean ± SEM. *P<0.05 and **P<0.01.

were also found in another NSCLC cell line, NCI-H292, in which the upregulation of SOCC components upon nicotine exposure were attenuted by siHIF-1α (data not shown).

HIF-1α deficiency abolishes the increases in basal $[Ca^{2+}]_i$ and SOCE induced by nicotine. Since HIF-1α deficiency reduced expression of SOCC components in A549 cells, we therefore measured the basal $[Ca^{2+}]_i$ and SOCE when HIF-1α expression was abolished. Downregulation of HIF-1α expression with siHIF-1α decreased basal $[Ca^{2+}]_i$ in the A549 cells and abolished the increase in basal $[Ca^{2+}]_i$ induced by nicotine (Fig. 5A and B). Furthermore, HIF-1α deficiency not only reduced SOCE in the A549 cells, but prevented SOCE increase induced by nicotine (Fig. 5A and C).

Loss of HIF-1α eliminates nicotine-induced cell proliferation. The effects of HIF-1α on cell proliferation were evaluated. As shown in Fig. 6, HIF-1α deficiency induced by siHIF-1α transfection suppressed cell proliferation in cells with or without nicotine exposure at 48 h.

Discussion

Lung cancer is a serious life-threatening disease and cigarette smoking is the primary risk factor. In the present study, we demonstrated that nicotine, the major component in cigarette smoke, upregulated the expression of HIF-1α and SOCC components, and promoted cell proliferation in A549 cells. Enhanced SOCE and elevated intracellular $[Ca^{2+}]_i$ were associated with the upregulation of HIF-1α. Silencing of HIF-1α or blocking SOCE abolished the nicotine-induced cell proliferation. Therefore, HIF-1α mediated the promotive effects of nicotine on SOCC component expression, SOCE and cell proliferation in lung cancer cells.

Intracellular $[Ca^{2+}]_i$ regulated by SOCE is essential for modulating cell migration and proliferation in normal and cancer cells. Suppression of SOCE by abolishing SOCC component expression was found to prevent the proliferation and invasion of lung cancer cells (28-30). In the present study, we determined the expression of SOCC components in A549 cells. The expression of TRPC isoforms in A549 cells is consistent with the expressional profile of TRPCs in NSCLC tissue, in which the mRNA levels of TRPC1, 3, 4 and 6 are detectable and the levels of TRPC2, 5 and 7 are below the detection limit (31).

Nicotine participates in the progression of lung cancer through the non-neuronal cholinergic system mediated by the nicotine acetylcholine receptor (32,33). Normal bronchial epithelial cells express α3-, α4-, α5- and α7-nAChR subunits which modulate Ca^{2+} metabolism (34). The receptors nAChRs are functionally conserved in mediating nicotine responses on TRPCs in neurons from worms to mammals (35). However, to date it is not known which nAChR subunit mediates the functions of nicotine in regulating expression of SOCC components in epithelial cells. The candidates could be α5- and α7-nAChR, as it has been suggested that the dysregulations of α5- and α7-nAChR in lung cancer tissues are associated with different influences of nicotine on the tumorigenesis of different lung cancer types (36).

The present study demonstrated that HIF-1α mediated the upregulatory effects of nicotine on TRPCs and Orai1. It has been confirmed that nicotine upregulates HIF-1α expression through binding α5-nAChR and activating the downstream Erk1/2 or PI3K/Akt signaling in NSCLC (26,37). Actually, the genes encoding TRPCs could be target genes of HIF-1α in modulating the growth of various types of cells, since, except for nicotine, HIF-1α is believed to be a common mediator of various factors in enhancing the expression of TRPCs, SOCC and intracellular $[Ca^{2+}]$ in pulmonary arterial smooth muscle cells and cardiomyocytes suffering hypoxia, which result in cell proliferation and migration or tissue hypertrophy (27,38,39).

Although the association between Orai1 dysexpression and lung cancer progression remains to be evaluated, Orai1 elevation is related to enhanced tumor cell proliferation and invasion (40). Our results not only demonstrated the involvement of Orai1 in nicotine-triggered lung cancer cell proliferation, but showed that *Orai1* may be a target gene of HIF-1α. Furthermore, like in other cell types, it is reasonable to suppose that the functions of Orai1 on SOCE in lung tumor cells might not be limited as a channel forming protein, but as a regulator for SOCC complex formation, including regulating the recruitment of TRPC1 (41) or the formation of ternary complex of TRPC-Orai1-STIM1 (42).

Based on our results, it is comprehensible to suggest that HIF-1α upregulation upon nicotine stimulation eventually promotes lung tumor cell proliferation by increasing expression of SOCC components and intracellular $[Ca^{2+}]$. Actually, nicotine-activated signaling mediated by PKC, NF-κB, Srk, PI3K/Akt, Raf-1, ERK1/2 and p90RSK are related with increases in cell proliferation, migration, invasion or inhibition of cell apoptosis (43-46). However, we did not observe an obvious change in migration within 24 to 48 h upon nicotine exposure, although inhibition of SOCE abolished cell migration (Fig. 3B and data not shown). Probably this was due to the lower concentration of nicotine used in this study when compared with other studies, which may need a longer time to trigger a change in migration capacity (25,47).

In summary, the present study demonstrated that nicotine upregulated the expression of SOCC components TRPC6 and Orai1 by increasing HIF-1α expression in NSCLC cells,

which eventually led to enhanced SOCE, elevated intracellular $[Ca^{2+}]_i$ and promotion of cell proliferation. These findings suggest that HIF-1α-SOCE signaling plays a pivotal role in pro-tumor functions of nicotine in NSCLC.

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Competing interests

The authors declare that they have no competing interests.

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