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Mitochondrial reactive oxygen species mediates nicotine-induced hypoxia-inducible factor-1α expression in human non-small cell lung cancer cells ☆

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ABSTRACT

Cigarette smoking is not only a documented risk for lung carcinogenesis but also promotes lung cancer development. Nicotine, a major component of cigarette smoke but not a carcinogen by itself, has been found to induce proliferation, invasion and metastasis of non-small cell lung cancer (NSCLC). Here we reported that proinvasive effect of nicotine is analogous to that of hypoxia and involves stabilization and activation of hypoxia-inducible factor (HIF)-1α, a key factor in determining the presence of HIF-1 and expression of its downstream metastasis-associated genes. Furthermore, nicotine-induced upregulation of HIF-1α was dependent on mitochondria-derived reactive oxygen species (ROS). Ectopic expression of mitochondrial targeted catalase effectively prevented nicotine-induced accumulation of HIF-1α protein. In addition, we demonstrated that the effect of nicotine in upregulation of HIF-1α was mediated by Dihydro-β-erythroidine (DhβE)-sensitive nicotine acetylcholine receptors (nAChRs) and required synergistic cooperation of Akt and mitogen-activated protein kinase (MAPK) pathways. These results suggest that exposure to nicotine could mimic effects of hypoxia to stimulate HIF-1α accumulation and activity that might underlie the high metastatic potential of lung cancer.

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1. Introduction

Lung cancer is leading cause of cancer-related death worldwide, with more than 1 million deaths every year [1]. Lack of effective tools for early diagnosis and highly metastatic potential might account for the marked lethality of lung cancer, with a overall 5-year mortality rate of still nearly 85% [2].

Cigarette smoke is the most established risk factor for lung cancer and is highly correlated with the onset of non-small cell lung cancer, which represents approximately 80% of all lung cancers [3]. In addition to its documented risks for lung carcinogenesis, cigarette smoking has been implicated in cancer development. However, the molecular mechanisms underlying its role in cancer progression are still poorly understood. Nicotine, a major component of cigarette smoke and not a carcinogen by itself, has been implicated in the progression and metastasis of lung cancer. Experimental evidence demonstrated exposure to nicotine and its derivatives like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) increases proliferation, invasion and epithelial-mesenchymal transition of NSCLC cell lines [4–6]. The

pathophysiological effects of nicotine and its derivatives are believed to be mediated by nicotinic acetylcholine receptors (nAChRs) in target cells, and in lung cancer predominantly by alpha7- and DhβE-sensitive subtypes of nAChRs [7,8]. The interaction of nicotine with nAChR has been found to increase oxidative stress and activate NF-κB and multiple signaling pathways that regulate the progression, growth, and metastasis of tumors [9,10].

Metastasis is a major step in disease progression and over 90% cancer deaths can be attributed to tumor metastasis [11]. It has been suggested that the acquisition of metastatic phenotype is achieved mainly through intratumoral hypoxia [12], which favor selection of more aggressive and invasive cells within tumor mass and therefore drive cancer progression [13]. To a large extent, many of these cellular responses to hypoxia are mediated by the transcriptional factor hypoxia-inducible factor 1 (HIF-1). HIF-1 consists of an inducible alpha subunit (HIF-1α) and constitutively expressed β subunit (HIF-1β), which heterodimerize and bind to hypoxia response elements (HREs) in promoter region of numerous metastasis-associated genes [14,15]. HIF-1α is continuously synthesized and degraded during normoxia. The degradation of HIF-1α is triggered by oxygen-dependent hydroxylation of two proline residues by a family of prolyl hydroxylase (PHD) enzymes. Aside from oxygen-dependent regulation of HIF-1α, the increase in HIF-1α levels is also regulated by growth factor stimulation and free radicals generation. Many lines of evidence indicate that levels of intracellular ROS were closely linked to HIF-1α stabilization possibly through modification

of PHD activity [14,16]. Furthermore, mitochondria, which are the main source of ROS production, appear to play a critical role in regulation of HIF-1α stability in such a way that mitochondrial targeted antioxidant prevents hypoxia-induced stabilization of HIF-1α protein [17].

The ability of nicotine to drive acquisition of malignant phenotype characterized by increased risk of metastasis is mostly similar for hypoxia, given the fact that development of metastases of lung cancer already started when the primary tumors are still small [2], we conjectured that exposure to nicotine might mimic the effects of hypoxia to stimulate HIF-1 activity and thus promote the progression of lung cancer. Indeed, a recent study showed that nicotine induced HIF-1α expression in NSCLC cell lines [18]. However, the molecular pathways underlying this observation have not been fully delineated. Therefore, it was the aim of the present study to identify signaling cascades and cellular processes that contribute to nicotine-induced HIF-1α activation.

2. Methods

2.1. Reagents, cells, and transfections

Nicotine, N′-Nitrosonornicotine (NNN), 4-methylnitrosamino-1-3-pyridinyl-1-butanone (NNK), Cobalt(II) chloride, and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO). The kinase inhibitors LY294002, PD98059, PP2, JNK inhibitor, and GSK-3β inhibitor were purchased from Calbiochem and used at concentrations of 20, 30, 30, 10, and 15 μM, respectively. All above concentrations were found to be effective in previous studies [19–21]. Rotenone, diphenyleneiodonium chloride (DPI), mecamylamine (MCA), methyllycaconitine (MLA) and dihydro-β-erythroidine hydrobromide (DhβE) were obtained from Sigma-Aldrich and concentrations of these inhibitors used in this study were referred to previous studies and titrated to effect without toxicity. [22,23]. Anti-Hif-1α antibody was obtained from Novus Biologicals (Littleton, CO), with anti-Hif-1β antibody obtained from BD (Franklin Lakes, NJ). Anti-COXIV, anti-pAkt, anti-Akt, anti-pErk, and anti-Erk antibody were purchased from Cell Signaling (Beverly, MA), with Anti-Mn SOD from Stress Gene (Victoria, BC), anti-human catalase from Athens Research Technology (Athens, GA). Human A549 cells, NL-9980 cells, NCI-H466 cells and YTMLC-9 cells were maintained in 1640 medium supplemented with 10% fetal bovine serum. For transfection, A549 cells were grown in six-well plates and transfected with Lipofectamine™ LTX purchased from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions.

2.2. DNA constructs

The full-length superoxide dismutase 2 (SOD2) gene was generated by PCR amplification using cDNA from A549 cells as template and primers: Forward: 5′-TAGAATTCATGTTGAGCCGGGCAGTGTGCGGCA-3′, reverse: 5′-CTAAGCTTTTACTTTTTGCAAGCCATGTATCTT-3′. The PCR products were digested with EcoRI and HindIII, and inserted into pcDNA3.1 expression vector (Invitrogen). The mitochondria target catalase plasmid was kindly provided by Dr. Peter S. Rabinovitch (Department of Pathology, University of Washington, Seattle).

2.3. Small interfering RNA (siRNA)

Chemically synthesized, double-stranded siRNA specifically target human HIF-1a 5′-CUGAUGACCAGCAACUUGA dTdT-3′ and 5′-UCAAGUUGCUGGUCAUCAG dTdT-3′ was obtained from Guangzhou Ribobio Co. Ltd (Guangzhou, China). To provide a control, a nontargeting siRNA was used for all experiments. A549 cells were plated in 6-well plates and when cells were 30–50% confluent, siRNAs were added in final concentration of 100 nM with lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions.

2.4. Cytotoxicity detection

Cells were seeded in triplicate in 12-well plates and treated with various concentrations of nicotine or 1% Triton X-100 as a positive control for 3 h. The medium was collected and lactate dehydrogenase (LDH) release into medium was detected by LDH cytotoxicity detection kit (Roche, Basel Switzerland) according to manufacturer's instructions. Data presented is a mean of 3 independent experiments.

2.5. Western blot analyses

A549 cells were seeded in 6-well plates (10^5 cells/well), and after various treatments they were lysed in pre-warmed Laemmli buffer purchased from Bio-Rad (Hercules, CA). For each sample the same amount of total protein was added to a well of 10% acrylamide gel and resolved by SDS-PAGE. A 1:800 dilution of anti-Hif-1α and Hif-1β, and a dilution of 1:1000 dilution of all other primary antibodies were used. Blots were quantified by densitometry using Transparency Adapter (UTA-2100XL) for PowerLook 2100XL obtained from UMAX (Mountain View, CA).

2.6. Determination of intracellular ROS levels

To measure the ROS, A549 cells were preincubated with 30 μM 2′,7′-dichlorofluorescein diacetate (DCFDA, Sigma), an efficient probe for identification of free radicals, especially H_2O_2 [24,25]. After 30 min, cells were harvested, washed and resuspended in PBS and incubated with indicated concentrations of nicotine incubation for 1 h. Fluorescence intensity was detected by flow cytometer (Cell Lab QuantaTMSC, Beckman Coulter).

2.7. Quantitative real time RT-PCR

Total RNA was isolated at the indicated time using the RNA isolation reagent TRIzol (Invitrogen). Reverse transcription was performed by using the Reverse Transcriptase M-MLV (RNase H⁻) kit (Takara Biotechnology, Dalian Co., Ltd). Each 25 μl of PCR reaction mixture was prepared using the SYBR Premix Ex Taq™ Kit (TaKaRa). The primer pairs used for each mRNA that was measured are shown in Table 1. The amplification conditions were according to the protocol of the SYBR Premix Ex Taq™ Kit. All the samples were performed in triplicates in each experiment in 7500 Real Time PCR System (Applied Biosystems, Foster city, CA). The relative amount mRNA was calculated using the comparative CT method after normalization to GAPDH mRNA levels.

2.8. Mitochondria isolations

A549 cells were plated in 6-well plates and transfection was done with indicated amounts of SOD2 and mitochondrial targeted catalase (MCAT) constructs using Lipofectamine™ LTX (Invitrogen). 72 h after transfection, cells was exposed to nicotine for additional 3 h and harvested. The mitochondria were isolated using the mitochondria

Table 1
Sequences of real-time RT-PCR primers.

Gene	Genbank accession number	Primer	PCR product (bp)
HIF-1α	NM_001530	Forward:5′-tgatgaccagcaacttgagg-3′ Reverse:5′-tggggcatggtaaaagaaag-3′	166
VEGF	NM_003376	Forward:5′-cccactgaggagtccaacat-3′ Reverse:5′-tttcttgcgctttcgtttt-3′	185
Glut-1	NM_006516	Forward:5′-cttcactgtcgtgtcgctgt-3′ Reverse:5′-tgaagagttcagccacgatg-3′	229
GAPDH	NM_002046	Forward:5′-gaccccttcattgacctcaac-3′ Reverse:5′-cttctccatggtggtgaaga-3′	195

isolation kit for culture cells according to instructions of the manufacturers (PIERCE, Rockford, IL).

2.9. Chromatin immunoprecipitation (ChIP) assay

We used the ExactaChIP™ human/mouse HIF-1α Kit (R&D, Minneapolis, MN) to get the target DNA. Briefly, A549 cells treated with nicotine or NNK were crosslinked with 1% formaldehyde and quenched by glycine. After washed with PBS, cells were scraped and collected by centrifugation and cell pellets were lysed with kit provided lysis buffer. The resulting nuclear pellet was sonicated (Vibra cell, SONICS, Newtown, CT) for four rounds of 15 pulses at 4% output power and 70% duty to obtain average 1 kb sheared chromatin. The sonicated lysates were used for immunoprecipitation with biotinylated antibodies either against HIF-1α or normal goat IgG. The immune complexes were recovered with magnetic streptavidin beads. The immunoprecipitated DNA and input DNA were extracted by incubation with chelating Resin solution and boiled for 10 min to reverse the cross-links. The DNA was purified with DNA purification kit (Qiagen). PCR was performed with purified DNA and recommended reaction condition. The primers for human $VEGF$ promoter are: forward: 5′-CCTCAGTTCCCTGGCAACATCTG-3′ and reverse: 5′-GAA-GAATTTGGCACCAAGTTTGT-3′.

2.10. Statistical analysis

Data are expressed as the mean ($\pm$ S.D.), and statistical comparisons were done by analysis of variance (ANOVA) with Dunnett's correction for multiple comparisons using SAS version 9 (SAS Institute Inc., Cary, NC) with $p < 0.05$ considered statistically significant.

3. Results

3.1. Nicotine increases levels of HIF-1α in a dose- and time-dependent manner

Nicotine was recently reported to induce HIF-1α expression in human NSCLC cells with 16 h exposure [18]. To explore the generality of mode and kinetics of HIF-1 regulation by nicotine, we examined the effect of nicotine on HIF-1 levels in NSCLC cell line A549 (human lung adenocarcinoma) across a range of concentrations and durations of exposure. The cells were rendered quiescent by serum starvation and subsequently treated with various concentrations of nicotine for 3 h. Previous studies have shown that A549 cells expressed the moderate basal levels of HIF-1α due to elevated glycolytic metabolism in cancer cells [26], however, HIF-1α levels were substantially elevated by nicotine treatment in a dose-dependent manner (Fig. 1A). The maximal induction of HIF-1α by nicotine was observed at 1 μM, which is physiologically relevant and can be detected in the serum of those who smoke 1 pack a day [4]. The stimulatory effect of nicotine on HIF-1α was not consistently observed at higher dose (5 μM). However, the reduced effect of nicotine at higher dose was not due to cytotoxicity as determined by analyzing lactate dehydrogenase release in culture medium. The LDH levels did not increase significantly upon 5 μM nicotine stimulation compared with low doses of nicotine treatment (Fig. 1B). The stimulatory effect of nicotine on HIF-1α protein expression was further corroborated by nicotine derivatives NNN and NNK. Exposure of A549 cells to either NNN or NNK led to a significant increase in HIF-1α levels at doses that were seen with nicotine-mediated induction (Fig. 1C). Furthermore, both NNK and NNN treated cells exhibited more potent response than nicotine treatment. To ensure our study relevant to lung cancer and not specific to a single cancer cell line, we examined the effect of nicotine on HIF-1α levels in a panel of human lung cancer cells that originated from different histological entity. The western blot of Fig. 1D showed that nicotine-induced HIF-1α expression was

also evident in NL-9980 (human large cell lung cancer), NCI-H466 (human small cell lung cancer) and YTMLC-9 (human lung squamous cell carcinoma) cells.

To determine the kinetics of HIF-1α expression regulated by nicotine, quiescent A549 cells were stimulated with 1 μM nicotine for different time periods; The robust increase in levels of HIF-1α was detected at the first time point of 3 h, which was continuously sustained over 8 h of nicotine stimulation, and then decayed as the duration of nicotine exposure increased to 24 h (Fig. 1E). Note treatment of A549 cells with nicotine under various conditions did not affect the levels of HIF-1β. The maximal increase in HIF-1α was observed with 1 μM nicotine applied for 3 h, therefore, this condition was chosen for experiments to elucidate the mechanism of nicotine-induced up-regulation of HIF-1α protein.

3.2. Nicotine stimulates HIF-1-dependent transcriptional activity

To determine whether nicotine-induced up-regulation of HIF-1α expression results in an increase in HIF-1 activity and HIF-1 regulated gene expression, we first performed quantitative real-time RT-PCR to analyze the expression of two known HIF-1 target genes, $VEGF$ and $glucose\ transporter-1$ ($Glut-1$). As expected, treatment of quiescent A549 cells with 1 μM nicotine showed a significant increase in $VEGF$ and $Glut-1$ mRNA levels (Fig. 2A). We noticed that the increase was statistically significant but quantitatively small, possibly due to high basal levels of HIF-1α protein in A549 cells. Interestingly, they were reduced upon treatment with 5 μM nicotine, in which $HIF-1α$ levels and its targeted genes mRNA expression showed faithful dose-dependent concordance. To confirm that nicotine-induced upregulation of $VEGF$ and $Glut-1$ were indeed regulated by HIF-1α, we employed RNA interference to silence HIF-1α expression in A549 cells. Fig. 2B showed a quantification of mRNA by real-time RT-PCR seventy-two hours after transfection of A549 cells with either siRNA targeting $HIF-1α$ or nontargeting control siRNA and subsequently exposure to nicotine for 3 h. Compared to cells treated with control siRNA, cells treated with HIF-1α siRNA showed a substantial decrease in $HIF-1α$ mRNA levels that was roughly 75% at 72 h post-transfection. Furthermore, knockdown of $HIF-1α$ transcripts by siRNA was also accompanied by a significant reduction of nicotine-induced mRNA levels of $VEGF$ and $Glut-1$. However, we noticed that the basal levels of individual HIF-1 target genes seem to differ in their response to $HIF-1α$ silencing. HIF-1α siRNA treated cells had significantly less $VEGF$ mRNA, but basal levels of $Glut-1$ mRNA were not altered by knockdown of $HIF-1α$, suggesting that the mechanisms for HIF-1-independent regulation of $Glut-1$ gene might be involved.

To further verify the direct involvement of HIF-1 in the observed induction of $VEGF$ expression by nicotine, ChIP assays were carried out in A549 cells exposed either 1 μM nicotine or 1 μM NNK. Immunoprecipitation with an antibody to HIF-1α revealed that HIF-1α antibodies specifically immunoprecipitated the region of $VEGF$ promoter covering HIF-1 binding site and both nicotine and NNK treatment significantly increased the recruitment of HIF-1 into $VEGF$ promoter compared with untreated cells (Fig. 2C). Taken together, these experiments indicated that up-regulation of HIF-1α by nicotine contributes to its physiological effects.

3.3. Antioxidant reduces HIF-1α levels in nicotine-treated cells

In order to determine the mechanism for nicotine-induced upregulation of HIF-1α, we first verify whether the effect of nicotine on HIF-1α levels was a consequence of change at mRNA expression levels analyzed by quantitative RT-PCR. The result showed no notable mRNA level changes of HIF-1α in A549 cells exposed to various concentrations of nicotine (Fig. 3A), suggesting the rate of synthesis is not responsible for the increased levels of HIF-1α induced by nicotine. HIF-1α is composed of a hypoxia-inducible subunit (HIF-1α)
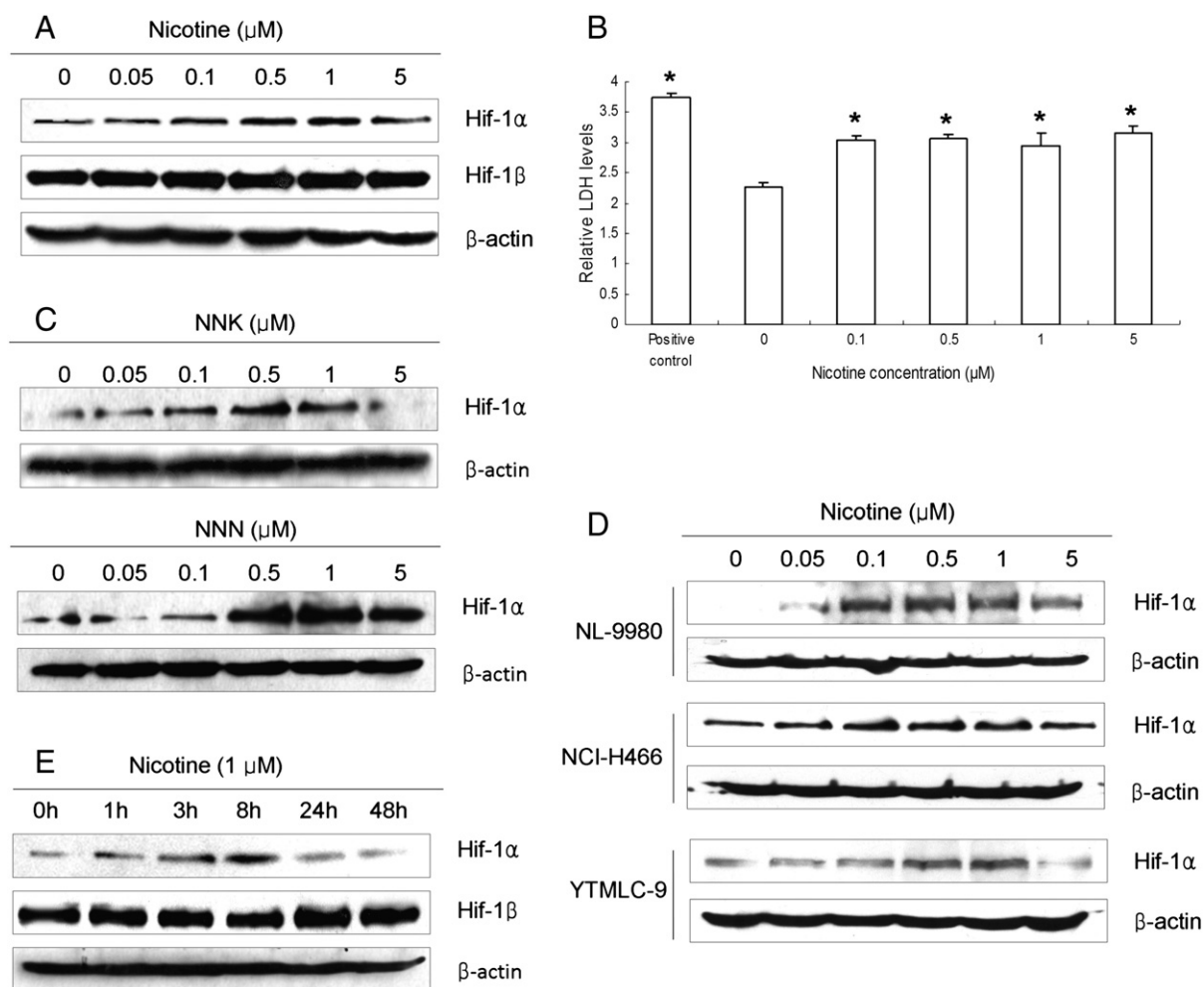
Fig. 1. Nicotine and its derivatives induce an increase in HIF-1α levels in a dose- and time-dependent manner. (A) Nicotine dose-dependently increased HIF-1α levels. A549 cells were stimulated without or with 0.05–5 μM nicotine for 3 h. (B) Nicotine did not significantly elicit cytotoxicity across a range of concentrations used. A549 cells were treated with different concentrations of nicotine or 1% Triton X-100 as positive control for 3 h. Culture medium was collected for analyzing the released lactate dehydrogenase (LDH). The bars represent the mean (± S.D.) calculated from three independent experiments done in triplicate. Although LDH levels increased upon nicotine treatment, compared with low doses of nicotine treated cells, the LDH levels did not increase significantly upon high doses of nicotine stimulation (*, $p < 0.05$ for difference from untreated control by ANOVA with Dunnett's correction for multiple comparisons). (C) Induction of HIF-1α levels by nicotine's derivatives NNK and NNN. A549 cells were treated by different concentrations of NNK (upper panel) or NNN (lower panel) for 3 h. Total cell lysates were subjected to western blot analysis using specific antibodies against HIF-1α. (D) Induction of HIF-1α by nicotine in different histological types of lung cancer cells. NL-9980 (human large cell lung cancer), NCI-H466 (human small cell lung cancer) and YTMLC-9 (human lung squamous carcinoma) cells were treated by different concentrations of nicotine for 3 h. Total cell lysates were subjected to western blot analysis with HIF-1α antibody. (E) Time-course of induction of HIF-1α by nicotine. A549 cells were treated without or with 1 μM nicotine for the indicated time and then lysed for western blot analysis using an antibody against HIF-1 α. The blot membrane was then detected for HIF-1β expression using anti-HIF-1β antibodies. Endogenous β-actin expression levels serve as internal controls.

and a constitutively expressed subunit (HIF-1β), and regulation of HIF-1 expression is mediated primarily through the post-translational stabilization of HIF-1α subunit in response to hypoxia. The similarity of pattern of HIF-1α induction by hypoxia and nicotine suggested that there might exist the common biological regulatory mechanisms between hypoxia and nicotine. To test this hypothesis, we incubated A549 cells with a combination of nicotine and a hypoxia-mimetic agent cobalt(II) chloride (CoCl₂). Treatment of quiescent A549 cells with CoCl₂ alone showed a clear dose-dependent increase in HIF-1α levels (Fig. 3B). However, a combination of CoCl₂ plus nicotine treatment exhibited the same pattern of HIF-1α levels as CoCl₂ alone, and nicotine did not additively enhance HIF-1α levels induced by CoCl₂ (Fig. 3C), implying two stimuli mediated their effects of HIF-1α induction through the common mechanisms. If each agent acting independently, one would have expected to see the minimum additive effects after co-treatment of nicotine and CoCl₂.

CoCl₂ was shown to mimic hypoxic response by inducing HIF-1α stability through the generation of increased ROS levels during normoxia [27]. To determine whether the ROS was involved in nicotine-induced upregulation of HIF-1α, A549 cells were pretreated with ROS scavenger N-acetyl-L-cysteine (NAC), and then exposed to nicotine for 3 h. As shown in Fig. 3D, HIF-1α induction by nicotine was significantly attenuated by antioxidant NAC in dose-dependent fashion. Similarly, NAC efficiently suppressed NNK-induced HIF-1α levels and eventually below the background levels (Fig. 3D, lower panel). Moreover, NAC pretreatment decreased nicotine-induced HIF-1α levels in various lung cancer cell lines (Fig. 3E), assuring the involvement of ROS in nicotine- and its derivative NNK-mediated upregulation of HIF-1α.

3.4. Mitochondrial ROS is required for nicotine-induced HIF-1α upregulation

Recently, the connection between mitochondrial ROS and HIF-1α stabilization has emerged [14,28]. In order to determine whether mitochondrial ROS could mediate nicotine-induced upregulation of HIF-1α, the cells were incubated with diphenylene (DPI), an inhibitor
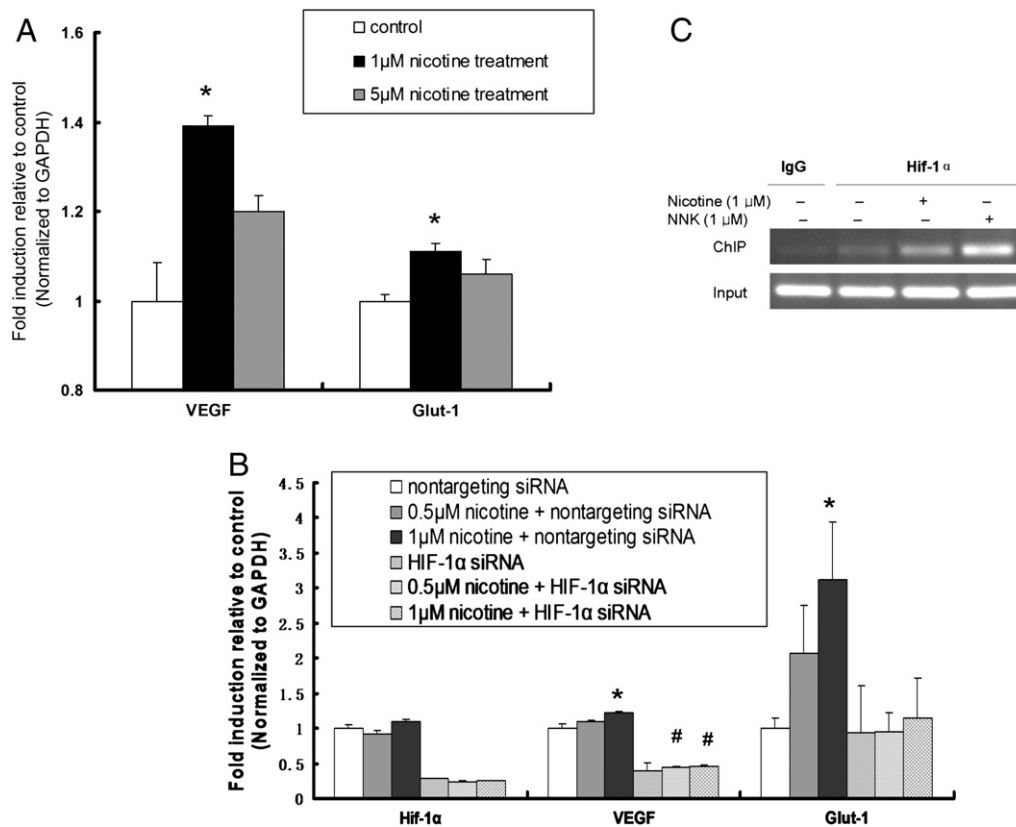
Fig. 2. Nicotine treatment induces HIF-1-dependent transcriptional responses. (A) Nicotine increases levels of two known HIF-1 target genes. A549 cells were exposed to different concentrations of nicotine (0, 1, 5 μM) for 3 h. Total RNA was isolated and cDNA was prepared. Quantitative real-time RT-PCR was done using primers for *VEGF* and *Glut-1* genes. Results are expressed as fold induction relative to cells without nicotine treatment after normalization to the GAPDH expression. The bars represent the mean ($\pm$S.D.) calculated from three experimental values. (*, $p < 0.05$ for difference from untreated control by ANOVA with Dunnett's correction for multiple comparisons). (B) Nicotine induces upregulation of *VEGF* and *Glut-1* is HIF-1 dependent. Forty-eight hours after transfection with HIF-1α or nontargeting control siRNA, A549 cells were placed on serum-free medium for 12 h and then incubated with different concentrations of nicotine (0, 0.05, 1 μM) for 3 h. Quantitative real-time PCR was performed for the analysis of mRNA expression levels of HIF-1α, VEGF and Glut 1 relative to internal control GAPDH. Reduction of HIF-1α levels significantly decreases the expression of V*EGF* and *Glut-1* mRNA. The bars represent the mean ($\pm$S.D.) calculated from three experimental values. (*, $p < 0.05$ for difference from untreated control by ANOVA and ($\#$, $p < 0.05$ for difference from HIF-1α siRNA treated cells by ANOVA with Dunnett's correction for multiple comparisons). (C) Nicotine and NNK increases binding of HIF-1 to *VEGF* promoter. A549 cells were incubated either with 1 μM nicotine or 1 μM NNK for 3 h and subjected to ChIP assays using anti-HIF-1 antibodies or control IgG. PCR using primers specific for *VEGF* promoter showed the predicted 205 bp band for each sample. There was a striking increase in the recruitment of HIF-1 to *VEGF* promoter by nicotine and NNK.

of NADPH oxidase enzymes that are major generators of non-mitochondrial ROS, or rotenone, an inhibitor of mitochondrial electron transport chain. As shown in Fig. 4A, nicotine increased HIF-1α levels in A549 cells, and this was not reduced by co-incubation with DPI, instead the nicotine-induced increase was slightly increased by 20 μM DPI. In contrast, the addition of rotenone dose-dependently abolished nicotine effect, suggesting mitochondrial ROS mediated nicotine-induced HIF-1α upregulation.

To genetically address the role of mitochondrial ROS nicotine-induced HIF-1α expression, transfection was performed to generate cells with increased levels of superoxide dismutase 2 (SOD2), which detoxifies superoxide to hydrogen peroxide (H_2O_2) in mitochondria, or mitochondrial catalase (MCAT), which is targeted to mitochondria for specifically scavenging mitochondrial H_2O_2 [29]. The cells were rendered quiescent 48 h after transfection and treated with 1 μM nicotine for 3 h. The efficiency of transfection was determined by preparing mitochondria fraction and by blotting either for COXIV, which is mitochondrial protein, or for SOD2 and MCAT. Dose-dependent increase of SOD2 levels strongly augmented the nicotine-induced HIF-1α levels (Fig. 4B), strikingly, cells transfected with a plasmid that expressed the mitochondria-targeted catalase (MCAT) showed marked and dose-dependent decrease in the levels of HIF-1α induced by nicotine (Fig. 4C). Taken together, the facts that the ability of nicotine to stimulate HIF-1α is enhanced by SOD2 but diminished by catalase indicated H_2O_2 is central to the regulatory

mechanism of nicotine-induced HIF-1α levels. Along these lines, the fluorescence probe DCFDA was used to monitor the intracellular generation of H_2O_2 in response to nicotine treatment [25]. Exposure of A549 cells to nicotine resulted in a substantial increase in fluorescent intensity in dose-dependent manner (Fig. 4F), confirming that the presence of increased H_2O_2 induced by nicotine.

3.5. Akt and MAPK signaling pathways linking mitochondrial ROS to HIF-1α accumulation

To further delineate the downstream signaling coupling mitochondrial ROS to the stabilization of HIF-1α, small molecule kinase inhibitors were employed to determine whether activation of Akt, MAPK, Src, JNK and GSK-3β, intracellular signaling pathways required for stabilization of HIF-1α during hypoxia [17], was involved in ROS-dependent stabilization of HIF-1α induced by nicotine. In contrast to Src inhibitor and JNK inhibitor and GSK-3β inhibitor, which had no effect, the PI-3 kinase inhibitor LY294002 and MAPK inhibitor PD98059 caused substantial reduction of HIF-1α expression in the presence of nicotine (Fig. 5A). The down-regulation of HIF-1α protein was not caused by non-specific effects of inhibitors because treatment of A549 cells with inhibitors alone, as shown in Fig. 5B, did not cause a discernable decrease in the levels of HIF-1α. Furthermore, LY294002 appeared to synergize with PD98059 action, HIF-1α levels were entirely abrogated by a combination of LY294002 and PD98059
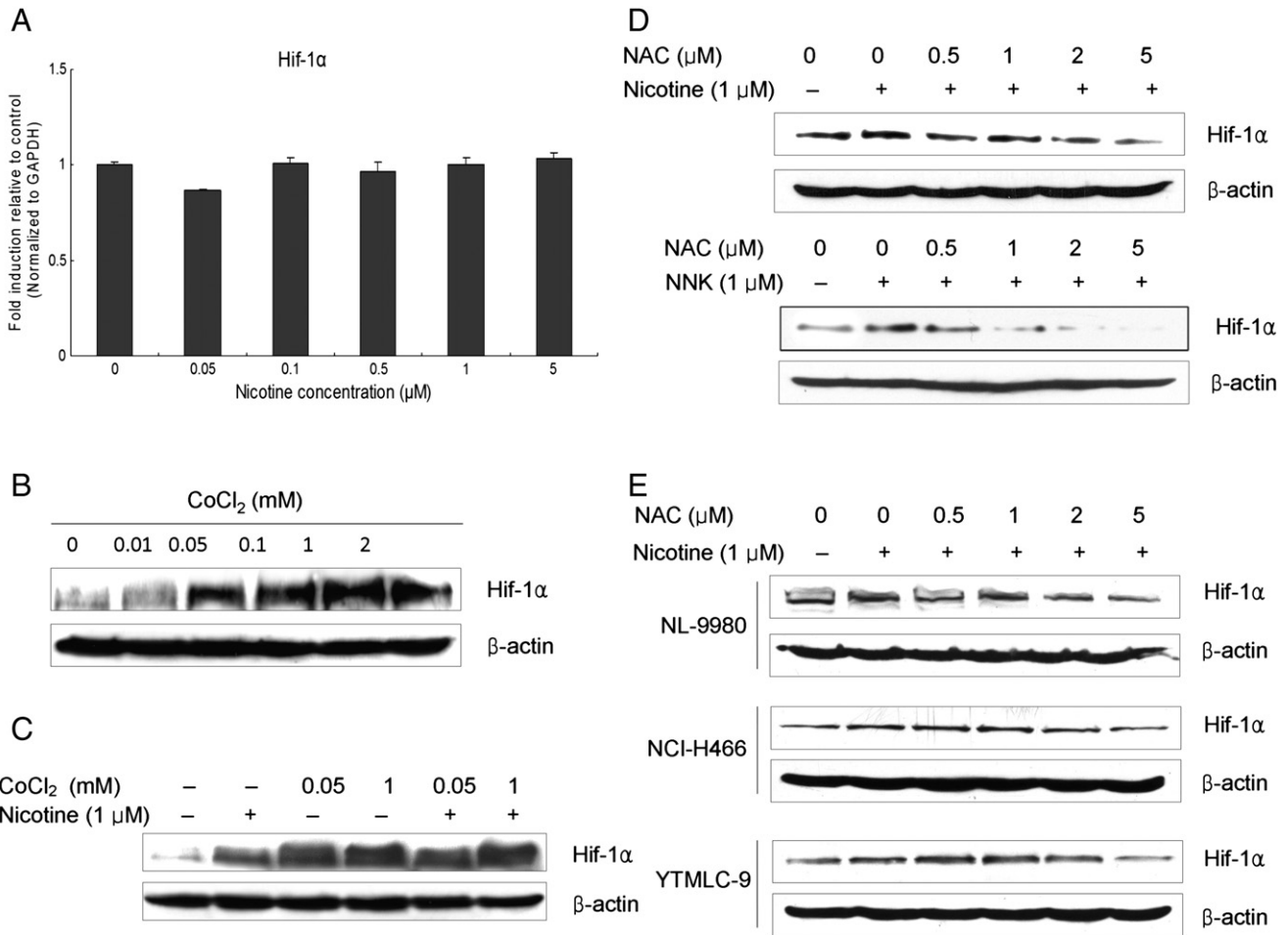
Fig. 3. Reactive oxygen species were involved in nicotine-induced upregulation of HIF-1α levels. (A) Quantitative real-time PCR showed nicotine treatment of A549 cells with various concentrations for 3 h did not affect HIF-1α RNA levels. The bars represent the mean ($\pm$ S.D.) calculated from three experimental values. (*, $p < 0.05$ for difference from untreated control by ANOVA with Dunnett's correction for multiple comparisons). (B) CoCl₂ stimulated HIF-1α protein expression in a dose-dependent manner. A549 cells were treated with increasing concentrations of CoCl₂ for 3 h before western blot analysis using an anti-HIF-1α antibody. (C) Nicotine had no additive effect with CoCl₂ on HIF-1α levels. Western blot analysis of HIF-1α levels showed nicotine did not additively enhance CoCl₂-induced HIF-1α expression in A549 cells exposed to nicotine (1 μM), CoCl₂ (0.05 and 0.1 mM) or nicotine (1 μM) plus CoCl₂ (0.05 and 1 mM) as indicated for 3 h. (D) Dose-dependent reduction of nicotine- and NNK-induced upregulation of HIF-1α levels by antioxidants. A549 cells were pretreated with increasing concentrations of N-acetylcysteine (NAC) for 30 min before 1 μM nicotine (upper panel) or 1 μM NNK (lower panel) was added. Western blot was conducted 3 h later to determine levels of HIF-1α. (E) Antioxidant NAC dose-dependently reduced HIF-1α levels in various lung cancer cell lines. NL-9980, NCI-H466 and YTMLC-9 cells were treated with 1 μM nicotine with and without pretreatment of indicated amounts of NAC for 30 min. HIF-1α levels were determined by western blot of total cell lysates.

treatment, suggesting Akt and MAPK pathways might be also required for maintaining a basal levels of HIF-1α expression. The participation of Akt and MAPK pathways in nicotine-induced HIF-1α levels was further supported by the fact that nicotine was able to stimulate Akt and MAPK activity, as indicated by enhanced phosphorylation of Akt and ERK (Fig. 5C, left panel). Interestingly, the most significant increase for both Akt and ERK phosphorylation was observed with 1 μM nicotine application, the same dose as for maximal stimulation of HIF-1α expression.

To extend our studies, we next asked if ROS could affect the ability of nicotine to stimulate Akt and MAPK pathways, A549 cells were pretreated with antioxidant NAC and then exposed to nicotine for 3 h. Antioxidant NAC dose-dependently decreased both Akt and ERK phosphorylation (Fig. 5C, right panel). To further confirm whether mitochondrial ROS mediates nicotine-dependent induction of Akt and MAPK activity, we ectopically overexpressed mitochondrial antioxidant enzymes SOD2 and catalase, as shown in Fig. 5D, transient transfection of SOD2 into A549 cells resulted in a dose-dependent increase in nicotine-induced phosphorylation of Akt and ERK (left panel), whereas dose-dependent increase of catalase levels significantly attenuated the levels of Akt and ERK phosphorylation induced by nicotine (right panel), revealing the similar effect of antioxidants

on nicotine-induced HIF-1α levels. Together, these results indicated mitochondrial ROS plays a critical role in nicotine-induced HIF-1α expression through Akt and MAPK pathways.

3.6. Involvement of DhβE-sensitive nAChR in nicotine-induced HIF-1α expression

Nicotine contributes to its physiological effects through stimulation of nicotinic acetylcholine receptors (nAChRs) in target cells. It was next examined whether the effect of nicotine on HIF-1α levels required nAChR function. Toward this purpose, A549 cells were preincubated with various receptor antagonists before stimulation with 1 μM nicotine. As shown in Fig. 6A and B, mecamylamine (MCA), a general antagonist of nAChR, and Dihydro-β-erythroidine (DhβE), an alpha4-specific nAChR antagonist, at 100 μM significantly reduced nicotine-induced upregulation of HIF-1α, whereas an antagonist of alpha7-specific nAChR methyllycaconitine (MLA) did not have any effect. To strengthen the above results, we examined the role of DhβE on nicotine-induced activation of Akt and MAPK pathways. Again, we observed a dose-dependent decrease of nicotine-induced phosphorylation of Akt and ERK by DhβE pretreatment (Fig. 6C). Taken
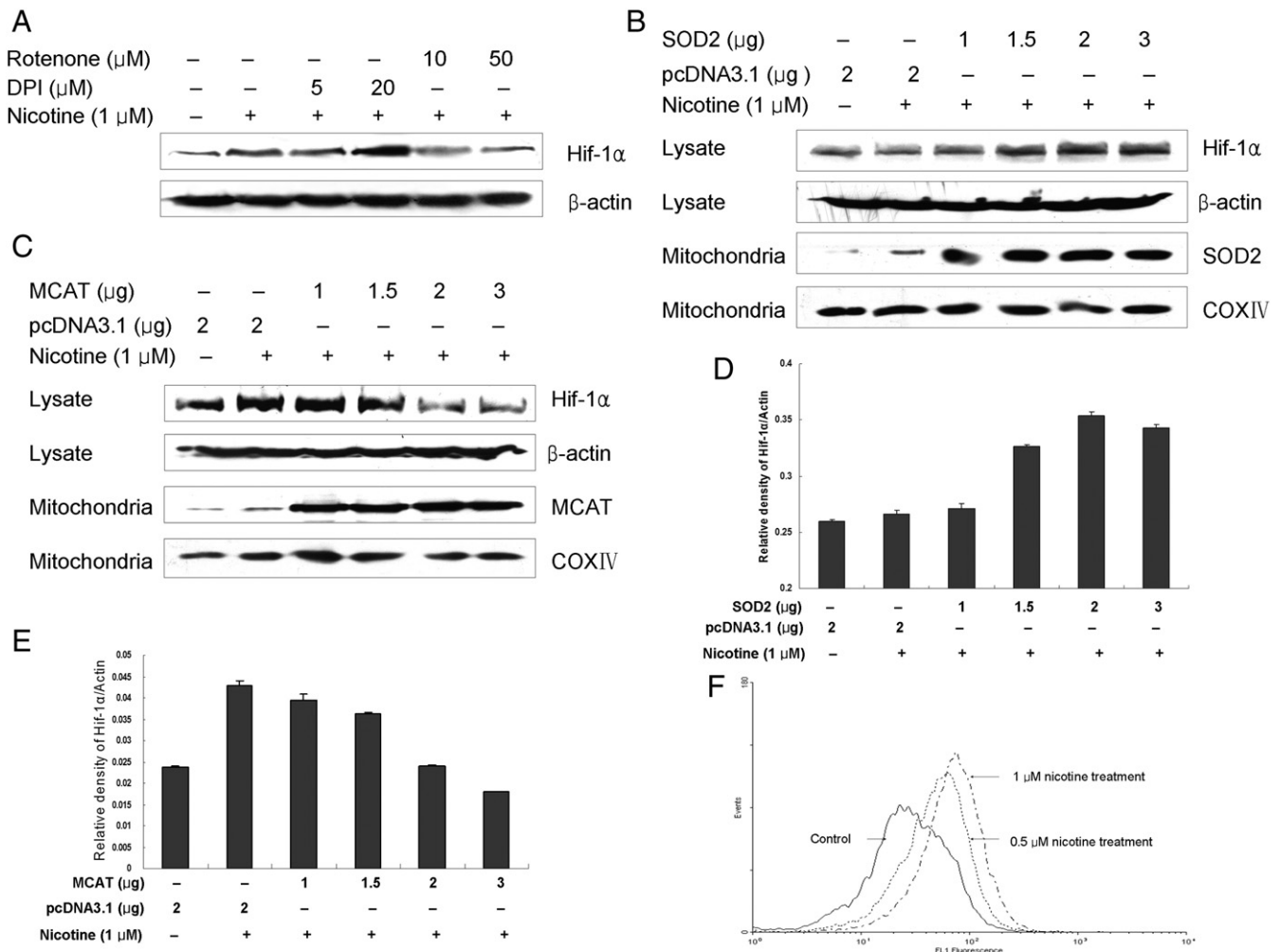
Fig. 4. Mitochondrial hydrogen peroxide mediated nicotine-induced HIF-1α levels. (A) The stimulatory effect of nicotine was inhibited by rotenone. A549 cells were pretreated with NADPH oxidase inhibitor DPI (5 and 10 μM) and mitochondrial ROS inhibitor, rotenone (10 and 50 μM) for 30 min. Cells were then stimulated with 1 μM nicotine in the absence and presence of inhibitors as above for 3 h. Western blot showed rotenone decreased nicotine-induced HIF-1α levels. (B and C) Hydrogen peroxide was required for accumulation of HIF-1α levels induced by nicotine 48 h after transfection either with indicated amounts of SOD2 expression plasmids (B) or the plasmid containing mitochondrial targeted catalase (MCAT) (C), A549 cells were starved with serum-free medium for 12 h and then incubated for 3 h with 1 μM nicotine. Mitochondria fractions were prepared and analyzed by western blot (bottom two panels) with an antibody against SOD2 (B), catalase (C) and mitochondrial protein COXIV (B and C) to determine the specificity and efficiency of transfection. Western blot was also performed with antibodies against HIF-1α using whole cell lysates (top two panels) prepared from the same treatment as above. Nicotine-induced HIF-1α levels was elevated in A549 cells upon transfection of SOD2, but was reduced in A549 cells transfected with catalase expression plasmid. (D and E) Densitometry confirmed that HIF-1α levels increased in A549 cells after transfected with increased amounts of SOD2 plasmids, but dose-dependently decreased in A549 cells transfected with mitochondrial targeted catalase (MCAT) expression vectors. (F) Representative histogram of DCF fluorescence. A549 cells were treated with indicated concentrations of nicotine for 1 h and evaluated for DCFDA as a measure of intracellular H_2O_2.

together, the induced effects of nicotine on HIF-1α in A549 cells are primarily mediated by DhβE-sensitive nAChR.

4. Discussion

Lung cancer is the most common cause of cancer-related deaths in the world. Development of metastasis significantly contributes to the high mortality of this disease. In many cases, the metastases already occurred by the time of diagnosis when the primary tumors are still small. Although the mechanisms of high metastatic potential of lung cancer remain unknown, mounting evidence support the idea that metastasis of lung cancer is probably influenced by intrinsic properties such as dysregulated signaling pathways as well as extrinsic properties such as communication with carcinogen-exposed micro-environment especially in the context of cigarette smoking [30]. However, most studies have focused on the role of smoking in lung carcinogenesis, the contribution of tobacco components to the lung cancer metastasis is relatively unexplored. Previous study showed that nicotine, an important component of cigarette smoking, can promote HIF-1α protein accumulation in a receptor-dependent manner [18], but the cellular processes mediating these events have not been fully identified. In the present study, we have shown that exposure to nicotine and its derivatives such as NNK can stimulate generation of mitochondrial ROS and mimic the effects of hypoxia to induce HIF-1α accumulation and activity. Moreover, the present study advances prior studies of nicotine by identifying Akt and MAPK as the key mediator of mitochondrial ROS stimulated by nicotine and upregulation of HIF-1α levels. These findings are likely to contribute to our understanding the role of nicotine in metastatic processes of lung cancer and give insight into novel therapies of lung cancer.

The role of HIF-1 in the development of metastatic diseases has long been recognized. Studies have shown that levels of HIF-1 increased with the progression of the pathological stage of tumors and have been positively related to tumor aggressiveness and a poor prognosis [31–34]. Aside from hypoxia, HIF-1α activity can also be induced or enhanced by growth factors stimulation and elevated reactive oxygen species (ROS) [14]. The recent discovery of mitochondria to regulate HIF-1α protein further underscores the
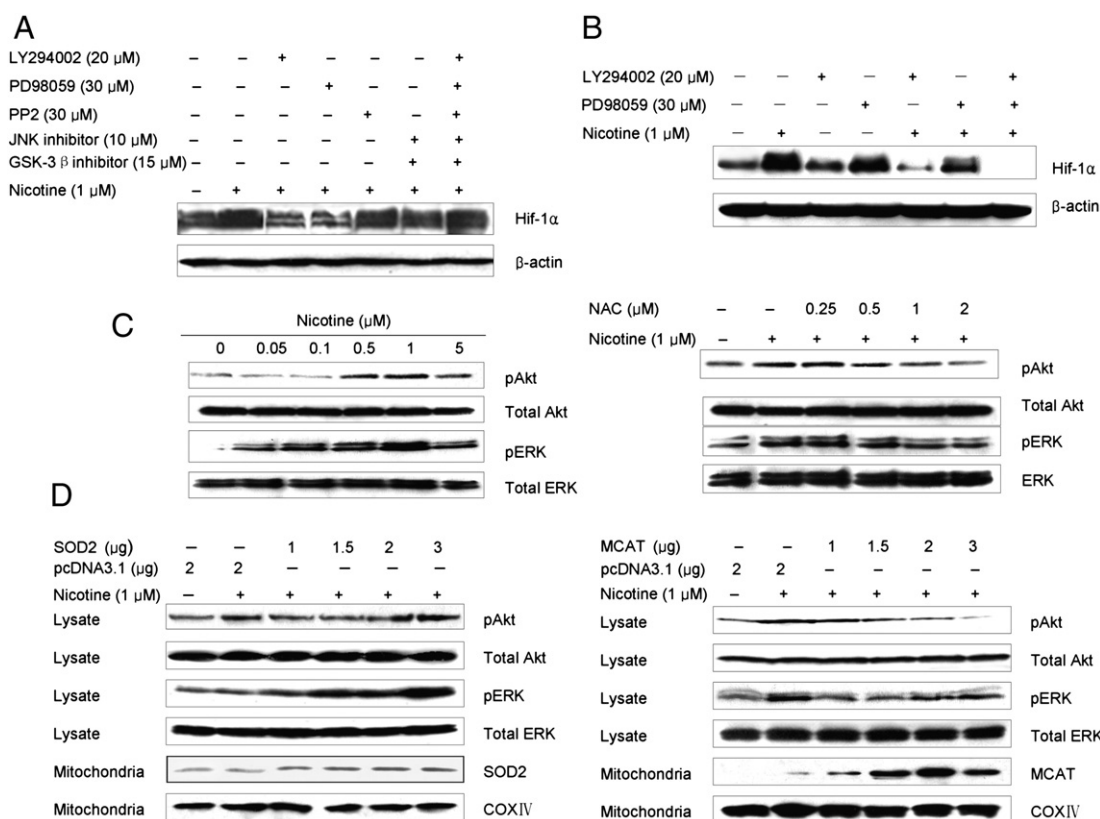
Fig. 5. Inhibition of Akt and MAPK pathways reduced nicotine-induced upregulation of HIF-1α. (A) Effect of kinases inhibitors on HIF-1α levels induced by nicotine. Total cell lysates were prepared from A549 cells cultured for 30 min in the presence of PI-3 kinase inhibitor (LY294002 at 20 μM), MEK inhibitor (PD98059 at 30 μM), Src inhibitor (PP2 at 30 μM), JNK inhibitor (JNK inhibitor at 10 μM), and GSK-3β-inhibitor (GSK-3β-inhibitor at 15 μM) before exposure of cells to nicotine (1 μM) for an additional 3 h, and analyzed by western blot with anti-HIF-1α. (B) Regulation of HIF-1α by LY294002 and PD98059. A549 cells were pretreated with kinases inhibitors, LY294002 (20 μM), PD98059 (30 μM) and LY294002 (20 μM) plus PD98059 (30 μM), for 30 min before treatment with 1 μM nicotine, and then subjected to western blot analysis for HIF-1α levels. LY294002 has an additive effect with PD98059 to further diminish HIF-1α levels. (C) Nicotine-induced activation of Akt and MAPK pathways was reduced by antioxidant N-acetylcysteine (NAC). Left panel indicated nicotine treatment induced activation of Akt and MAPK signaling pathways in a dose-dependent manner. A549 cells were treated with indicated concentrations of nicotine for 30 min. Whole cell lysates were prepared and analyzed for total protein levels and phosphorylated form of Akt and ERK, respectively. (Right panel) Total lysates were obtained from A549 cells pretreated for 30 min with different concentrations of NAC before 1 μM nicotine was added. Western blot was conducted with antibodies against total- or phosphorylated-Akt and ERK. (D) Catalase and SOD2 modulated nicotine-stimulated activation of Akt and MAPK pathways. A549 cells transfected either with SOD2 expression plasmid (1, 1.5, 2, 3 μg) (left panel), or plasmid containing mitochondrial targeted catalase (1, 1.5, 2, 3 μg) (right panel) for 48 h prior to serum starvation and stimulation with 1 μM nicotine. Mitochondrial fraction (bottom two panels) and total cell lysates (top four panels) were prepared and analyzed by western blot with antibodies against indicated proteins.

essential contribution of ROS in accumulating HIF-1α levels [35,36]. Initial studies from Mansfield et al. demonstrated that cells are not able to stabilize HIF-1α protein during hypoxia when knocking out the cytochrome c gene [35]. Complimentary findings using RNA interference have also shown that knockdown of the complex III subunit Rieske iron sulfur protein (RISP) effectively prevents hypoxic stabilization of HIF-1α protein [36], indicating mitochondrial electron transport chain is necessary for stabilizing HIF-1α during hypoxia. Paradoxically, mitochondrial electron transport chain increases the production of ROS during hypoxia and these mitochondria-derived ROS appear to be both necessary and sufficient to stabilize HIF-1α protein [17]. Indeed, studies from McCreath et al. showed cells treated with the mitochondrial targeted antioxidant MITOQ result in failure to stabilize the HIF-1α protein during hypoxia [37]. These observations lend strong support for a role of mitochondrial ROS in stabilization of HIF-1α protein.

Multiple lines evidence indicate that nicotine increases oxidative stress associated with cardiovascular, pulmonary and neoplastic disorders [9]. In addition, nicotine has been shown to cause oxidative stress to the pancreatic tissue of rats as demonstrated by increasing free radical generation and lipid peroxidation [38]. However, the results of our study highlighted the essential role of mitochondrial ROS induced by nicotine in triggering stabilization of HIF-1α. How mitochondrial ROS is generated by nicotine is another important issue. Previous studies observed the inhibitory effect of nicotine on

mitochondrial respiratory chain [39,40]. This may be of potential relevance taking in mind that disruption of mitochondrial respiratory chain is likely to increase free radical production due to leakage of electrons from respiratory complexes, an event that has been described to occur under hypoxia condition [17]. Along these lines, a recent study showed that neonatal exposure to nicotine significantly increases pancreatic ROS generation and protein carbonylation, particularly within mitochondrial fraction [41]. Nicotine exerts its biological effects mainly through stimulation of the nicotinic acetylcholine receptors (nAChRs), a family of ligand-gated Ca^{++} or Na^+ ion channel receptors that mediate nicotine addiction in brain and proliferation, apoptosis, and migration of non-neuronal cells. Binding of nicotine to nAChRs leads to increased levels of intracellular Ca^{++}, which in turn causes Ca^{++} flux into mitochondria [8,9]. High levels of Ca^{++} in the mitochondria activate mitochondrial metabolism. There might be some mechanistic link between a rise in mitochondrial Ca^{++} concentration and an increase in ROS generation. The molecular basis by which elevated Ca^{++} concentration increases mitochondrial ROS production is worthy of future investigation.

Interestingly, in accordance with previous reports [4,42], our results indicated nicotine-induced HIF-1α levels was indeed mediated by DhβE-sensitive nicotine acetylcholine receptors (nAChRs). However, studies from other labs also indicated the important role of α7-nAChRs in mediating the pathophysiological effects of nicotine [5,43]. Although such a discrepancy is not entirely clear at present,
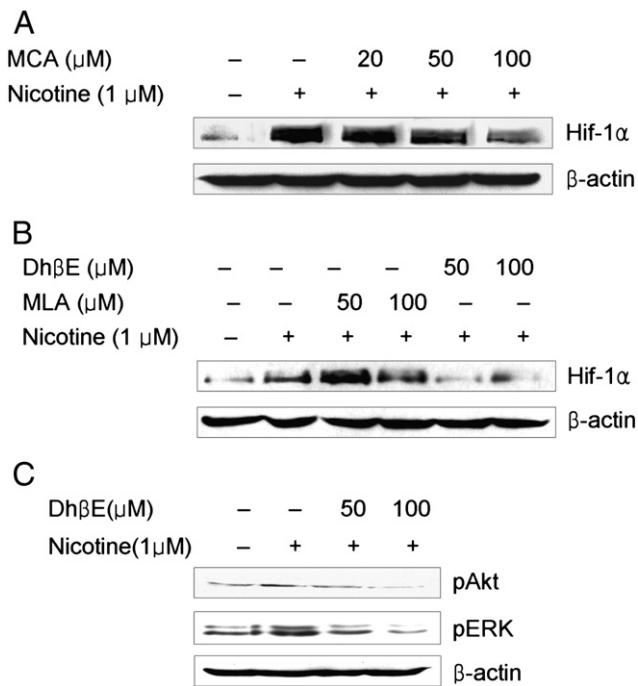
A

MCA (µM)   –   –   20   50   100
Nicotine (1 µM) –   +   +   +   +

Hif-1α

β-actin

B

DhβE (µM)   –   –   –   –   50   100
MLA (µM)   –   –   50   100 –   –
Nicotine (1 µM) –   +   +   +   +   +

Hif-1α

β-actin

C

DhβE(µM)   –   –   50   100
Nicotine(1µM) –   +   +   +

pAkt

pERK

β-actin

Fig. 6. The effect of nicotine on HIF-1α.expression was mediated via DhβE-sensitive nAChR. (A and B) A549 cells were pretreated with the increasing concentrations of mecamylamine, a general antagonist of nAChR (A), or with DhβE (50 µM and 100 µM), α3- and α4-specific nAChR antagonist, or MLA (50 µM and 100 µM), α7-specific antagonist (B), before 1 µM nicotine was added. Western blot was conducted 3 h later to determine HIF-1α levels. (C) Dose-dependent reduction of nicotine-induced Akt and ERK activation by DhβE. A549 cells were pretreated with indicated amounts of DhβE for 30 min before 1 µM nicotine was added. Blot was probed with antibodies against phospho-Akt and phospho-ERK.

it has been hypothetically suggested that proliferative effects of nicotine are mediated by α7-nAChRs, whereas DhβE-sensitive nicotine acetylcholine receptors mediates pro-survival effects of nicotine [8]. This may be relevant because elevated HIF-1α has been found to resist to apoptosis by upregulation pro-survival gene expression [44]. Whether the pro-survival effects of nicotine are mediated through HIF-1 deserves future study.

The specific signaling pathways linking mitochondrial ROS to HIF-1α accumulation have also been addressed. In the present study, the possibility that activation of Akt and MAPK pathways is required for nicotine-induced HIF-1α stabilization was validated in two ways. First, the blockade of Akt and ERK activation by kinases inhibitors greatly decreased nicotine-induced HIF-1α levels. Moreover, the levels of HIF-1α were totally abolished by combined PD98059 plus LY290042 treatment, suggesting the requirement of synergistic cooperation between Akt and MAPK pathways for nicotine-induced HIF-1α. Second, nicotine-induced activation of Akt and ERK was inhibited by mitochondrial catalase but enhanced by SOD2, the same pattern as nicotine-induced HIF-1α levels. Taken together, our results support a major role of Akt and MAPK pathways in nicotine-induced HIF-1α in NSCLC cell line. However, the possibility of indirect involvement of other signaling pathways such as NF-κB and Src cannot be ruled out in our study. The mechanisms of how Akt and MAPK prevent hydroxylation of HIF-1α protein remain unknown.

The data reported herein that nicotine can mimic effects of hypoxia to increase the mitochondrial ROS production and in turn upregulate HIF-1α protein not only provide delineation of molecular mechanisms underlying the high metastatic potential of lung cancer but also a strong rationale for the development of novel approach targeting the mitochondrial ROS to prevent HIF-1α activation. The significant work has recently been reported using three tumorigenic animal models that antitumorigenic effects of antioxidants in vivo are indeed HIF-1 dependent [45], strongly supporting the potential application of antioxidants in combating HIF-1 dependent NSCLC. However, the mitochondrial targeted antioxidants that scavenge ROS within mitochondria and do not affect the redox status of cytosol might be a more valuable tool to ablate HIF-1 activity. Our results not only established an important role of nicotine and its derivatives in the development of lung cancer but also open a novel avenue for therapy of cancers promoted by smoking.

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