β_2-adrenergic receptor activation promotes the proliferation of A549 lung cancer cells via the $ERK_{1/2}$/CREB pathway

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Abstract. Lung cancer is one of the most common cancers worldwide and accounts for 28% of all cancer-related deaths. The expression of the β_2-adrenergic receptor (β_2-AR), one of the stress-inducible receptors, has been reported to be closely correlated with malignant tumors. However, the role of β_2-AR activation in human lung epithelial-derived cancer A549 cells and the underlying mechanisms are not fully understood. In the present study, we found that activation of β_2-AR but not β_1-AR promoted the proliferation of A549 cells. Isoproterenol (ISO) stimulation of β_2-AR induced extracellular signal-regulated kinase 1/2 ($ERK_{1/2}$) and cyclic adenosine monophosphate response element-binding protein (CREB) phosphorylation. Blocking the $ERK_{1/2}$ pathway by U0126 inhibited CREB phosphorylation and also suppressed A549 cell proliferation. Moreover, ISO treatment enhanced the expression of matrix metalloproteinase (MMP) family proteins such as MMP-2, MMP-9, and also vascular endothelial growth factor (VEGF), which were able to be blocked by knockdown of CREB. In conclusion, our data revealed that β_2-AR induced $ERK_{1/2}$ phosphorylation which in turn activated CREB to promote A549 cell proliferation. These findings elucidate potential therapeutic targets for lung cancer treatment.

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Abbreviations: β_2-AR, β_2-adrenergic receptor; ISO, isoproterenol; $ERK_{1/2}$, extracellular signal-regulated kinase 1/2; CREB, cyclic adenosine monophosphate response element-binding protein; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; HPA, hypothalamic-pituitary-adrenal; ICI, ICI-118,551; EGFR, epithelial growth factor receptor; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase

Key words: β_2-adrenergic receptor, isoproterenol, proliferation, $ERK_{1/2}$, CREB, lung cancer

Introduction

Lung cancer is considered as the leading cause of cancer-related mortality among men worldwide and the second among women (1). Almost as many patients die of lung cancer every year than die of prostate, breast and colon cancer combined in the USA (2). According to the differences in clinical behavior and the aims of treatment, lung cancers can be divided into two broad categories; small cell lung cancer (SCLC) which accounts for 15% of all lung cancer cases and non-small cell lung cancer (NSCLC) which accounts for 85% of cases (3).

A number of studies have documented smoking as the leading risk factor for lung cancers. Since the first Surgeon General's Report on the association of smoking with lung cancer in 1964, increased public awareness of the health risks associated with smoking has gradually decreased the number of smokers over the past five decades in developed countries (4). However, contrary to expectations, the significant decrease in smokers has not yielded a correlating decrease in the incidence and mortality of lung cancer (5); this finding strongly suggests that factors other than smoking play significant roles in the development, progression and responsiveness to therapeutics of lung cancer. Interestingly, increasing evidence suggests that chronic psychological stress has been recognized as an important risk factor in promoting the genesis and development of cancers including lung cancer in recent years (6). In response to stressors, activation of the hypothalamic-pituitary-adrenal (HPA) axis leads to the release of glucocorticoids, catecholamines, including norepinephrine and epinephrine, and other stress hormones from the adrenal gland as well as from the brain and sympathetic nerve terminals (7). The effects of catecholamines are mainly mediated by β-adrenergic receptors (β-ARs) which are expressed in almost all mammalian cell types. Recent findings have indicated that β-ARs can regulate multiple cellular processes that contribute to the initiation and progression of cancer, including inflammation, angiogenesis, apoptosis, cell motility and trafficking, and the activation of tumor-associated viruses (8).

β-ARs, consisting of three subtypes (β_1-AR, β_2-AR and β_3-AR), are members of the superfamily of G protein-coupled receptors (GPCRs). β_1-ARs and β_2-ARs are expressed in the majority of mammalian cells, while β_3-ARs are almost exclusively found in adipocytes (9). β_2-ARs can associate with heterotrimeric guanine nucleotide-binding proteins

(G proteins), and multiple cytosolic scaffold proteins including β-arrestin and Src, to initiate various signaling pathways and modulate the activity of intracellular effectors such as adenylyl cyclase and mitogen-activated protein kinases (MAPKs) (10). Studies have shown that nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) binds to the β_1-AR-induced extracellular signal-regulated kinase 1/2 ($ERK_{1/2}$) and cyclic adenosine monophosphate response element-binding protein (CREB)/ATF-1 phosphorylation via transactivation of the epithelial growth factor receptor (EGFR) pathway in both human lung adenocarcinoma cell line NCI-H322 and human peripheral airway cell line HPLD1 (11). Moreover, NNK also induced $ERK_{1/2}$ and CREB phosphorylation through β_2-AR in pulmonary adenocarcinoma in the hamster (12). However, the function of β_2-AR in human lung epithelial-derived A549 cells and the underlyng mechanisms are not well understood.

To this end, the present study was designed to investigate the function and mechanism of β_2-AR-mediated cell proliferation in A549 cells. Our results demonstrated that activation of the β_2-AR can significantly enhance the proliferation of lung cancer cells via activation of the $ERK_{1/2}$/CREB pathway. In addition, matrix metalloproteinase (MMP)-2, MMP-9 and vascular endothelial growth factor (VEGF) may be involved in isoproterenol (ISO)-induced A549 cell proliferation.

Materials and methods

Antibodies and reagents. Antibodies for western blotting, including phospho-$ERK_{1/2}$ (Thr202/Tyr204) (cat. 9101, polyclonal, raised in rabbit, 1:2,000), $ERK_{1/2}$ (cat. 9102, polyclonal, raised in rabbit, 1:2,000), phospho-CREB (Ser133) (cat. 9198, monoclonal, rabbit anti-human, 1:2,000), CREB (cat. 9197, monoclonal, rabbit anti-human, 1:2,000), β-actin (cat. 4970, monoclonal, rabbit anti-human, 1:4,000), anti-rabbit IgG HRP-linked antibody (cat. 7074, goat anti-rabbit, 1:20,000) as well as U0126 (specific $ERK_{1/2}$ inhibitor) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). ISO (a broad-spectrum β-adrenergic agonist) and ICI-118,551 (ICI) (a selective β_2-AR antagonist) were purchased from Tocris Cookson, Inc. (Bristol, UK). Fetal bovine serum (FBS), culture medium and other solutions used for cell culture were from Invitrogen (Shanghai, China). MTT was purchased from Carl Roth GmbH & Co., KG (Karlsruhe, Germany). SYBR® Premix Ex Taq™ II was obtained from Takara Bio, Inc. (Otsu, Japan). Lipofectamine 2000 was procured from Thermo Fisher Scientific (Shanghai, China). The siRNAs against CREB and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and transfection. Human lung adenocarcinoma A549 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to the relevant specifications. The cells were grown in Dulbecco's minimal essential medium (DMEM) containing 4.5 g/l glucose supplemented with 10% FBS and 1% glutamine. All cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. A549 cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions, using siRNAs against CREB for 48 h.

Reverse transcription PCR. Total RNA was isolated from the A549 cells using TRIzol reagent, and reverse transcription was carried out according to the manufacturer's instructions from Invitrogen. PCR amplification was performed on 5 μl cDNA, 12.5 μl 2X Taq PCR Master Mix, 0.5 μl of 10 μM forward primer, 0.5 μl of 10 μM reverse primer and 6.5 μl ddH_2O under the following conditions: 94˚C for 5 min, 30 cycles of denaturation for 1 min at 95˚C, 1 min of annealing at 55˚C, elongation at 72˚C for 1 min, and extension at 72˚C for 1 min. PCR analysis was performed using the following sense and antisense primers: β_1-AR forward, 5'-GGG AGA AGC ATT AGG AGG G-3' and reverse, 5'-CAA GGA AAG CAA GGT GGG-3' which amplify a 270-bp fragment; β_2-AR forward, 5'-CAG CAA AGG GAC GAG GTG-3' and reverse, 5'-AAG TAA TGG CAA AGT AGC G-3' which amplify a 334-bp fragment; β-actin forward, 5'-ACA ACT TTG GTA TCG TGG AAG G-3' and reverse, 5'-GCC ATC ACG CCA CAG TTT C-3' which amplify a 101-bp fragment; MMP-2 forward, 5'-CCG TCG CCC ATC ATC AAG TTC-3' and reverse, 5'-GCA GCC ATA GAA GGT GTT CAG G-3' which amplify a 90-bp fragment; MMP-9 forward, 5'-TGG TCC TGG TGC TCC TGG TG-3' and reverse, 5'-GCT GCC TGT CGG TGA GAT TGG-3' which amplify a 111-bp fragment; VEGF forward, 5'-CTG GGC TGT TCT CGC TTC G-3' and reverse, 5'-CTC TCC TCT TCC TTC TCT TCT TCC-3' which amplify a 140-bp fragment.

Quantitative real-time polymerase chain reaction (RT-qPCR). The cDNAs were obtained and primers were used as previously described (13). The analysis using SYBR® Premix Ex Taq™ II from Takara Bio, Inc., the 20 μl PCR system was composed of 10 μl SYBR® Premix Ex Taq™ II, 0.4 μl ROX Reference Dye II, 6 μl ddH_2O, 0.8 μl of 10 μM forward primer, 0.8 μl of 10 μM reverse primer and 2 μl cDNA. RT-qPCR amplification was performed under the following conditions: 95˚C for 30 sec, 40 cycles of denaturation for 5 sec at 95˚C, 34 sec of annealing at 60˚C, elongation at 95˚C for 15 sec, and extension at 60˚C for 1 min. At least three independent experiments were conducted and samples were assessed in triplicate in each experiment.

Western blotting. Lysates from the cultured cells were sonicated and protein concentrations were determined using the Bradford reagent from Bio-Rad Laboratories. Equal amounts of protein (20 μg) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), which were incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline and 0.1% Tween-20) for 1 h, followed by incubation with the primary antibodies overnight at 4˚C and a 2-h incubation with HRP-conjugated secondary antibodies (1:20,000). Signals were visualised by enhanced chemiluminescence (Pierce, Rockford, IL, USA) associated fluorography, and their quantification was conducted by volume densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and total protein normalization.

MTT assay. Cell proliferation was investigated by MTT assay. Briefly, the cells ($5x10^4$/well) were plated into flat-bottom 24-well plates (Costar, Corning, NY, USA). After 24 h,

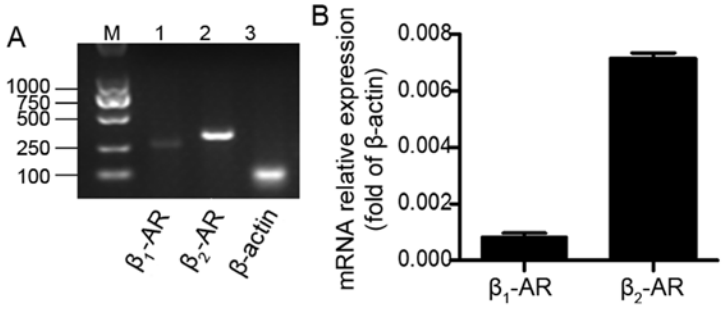

Figure 1. Expression of β_1-AR and β_2-AR at the mRNA levels in the A549 cells. Expression of mRNAs for β_1-AR, β_2-AR, and β-actin by (A) reverse transcription PCR and (B) RT-qPCR. Data are shown as means ± SEM of three triplicate experiments. β_2-AR, β_2-adrenergic receptor; RT-qPCR, quantitative real-time polymerase chain reaction.

the cells were serum-starved overnight and incubated with different concentrations of ISO and U0126 with or without ICI for different times. Following incubation, 20 μl MTT (5 mg/ml) was added to each well, and the cells were grown in complete media at 37˚C for 3 h. The supernatant was removed, and then 500 μl DMSO was added to each well of the 24-well plate and oscillated for 10 min. Subsequently, the absorbance was read at 570 nm using an enzyme-linked immunosorbent assay reader.

Statistical analysis. Data are presented as means ± SEM from at least three independent experiments. Statistical analysis of the data was performed using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

β_1-AR and β_2-AR expression in lung cancer cells. We first detected whether lung cancer cells express β-ARs. The results of reverse transcription-PCR analysis indicated that both β_1-AR and β_2-AR were expressed in the A549 cells (Fig. 1A). This was confirmed by RT-qPCR analysis (Fig. 1B). Interestingly, the levels of β_2-AR mRNA were significantly higher as compared to β_1-AR. This result suggests that β_2-AR may be the predominant β-AR in A549 cells and this finding encouraged us to elucidate the role of β_2-AR in these cells.

β_2-AR activation promotes the proliferation of human A549 cells. We then investigated the effect of β-AR agonist ISO on the A549 cell proliferation. We first analyzed the dose-dependent effect of ISO on A549 cell viability. The A549 cells were incubated with 0.1, 1, 5, 10, 20 and 30 μM ISO for 24 h and then cell viability was detected by MTT assay. As shown in Fig. 2A, ISO significantly enhanced the cell viability of the A549 cells maximally by a single treatment with 10 μM ISO while cell viability decreased dramatically with higher doses of ISO. To demonstrate the effects of ISO at different treatment times on A549 cell viability, the cells were incubated with 10 μM ISO for 24, 48 or 72 h. Cell viability assays revealed that ISO significantly increased A549 cell growth at these three time points with the strongest effect at 48 h (Fig. 2B). Based on these results, we chose 10 μM ISO as the reference concentration and a 48-h treatment time for the subsequent studies unless specifically indicated. Importantly, pre-treatment of the A549 cells with the β_2-AR-specific antagonist ICI to block

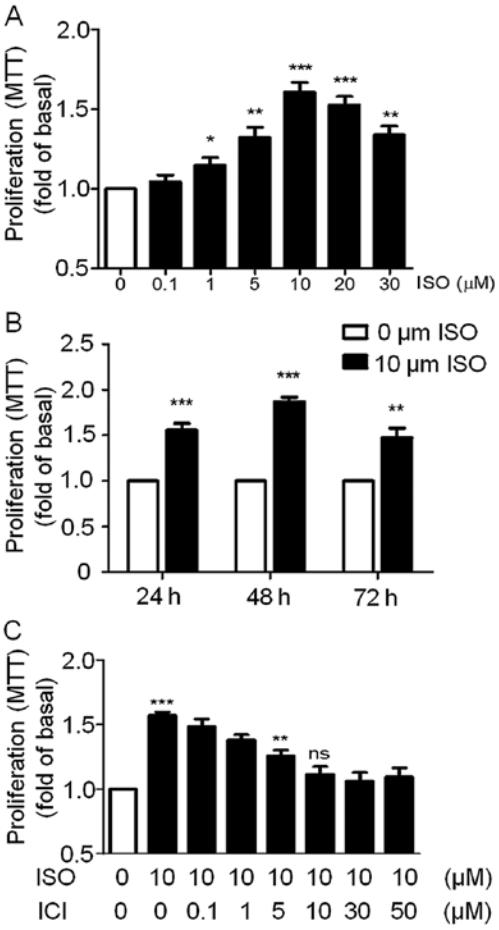

Figure 2. ISO promotes the proliferation of A549 cells. (A) Cells were starved overnight and then treated with increasing doses of ISO as indicated above for 48 h. Cell viability was determined by MTT assay. (B) Cells were treated with 10 μM ISO for 24, 48 and 72 h. (C) Cells were pre-treated with different concentrations of ICI for 30 min, and then incubated with 10 μM ISO and ICI for 48 h to measure the cell viability. Data are shown as means ± SEM from at least three triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, ns vs. control. ISO, isoproterenol; ICI, ICI-118,551; ns, not significant.

endogenous β_2-ARs blocked ISO-induced cell growth in a dose-dependent manner and was completely blocked following a 10 μM dose (Fig. 2C). These results suggest that ISO was able to promote A549 cell proliferation through activation of β_2-AR. This is consistent with our previous data showing that β_2-AR is highly expressed in A549 cells (Fig. 1).

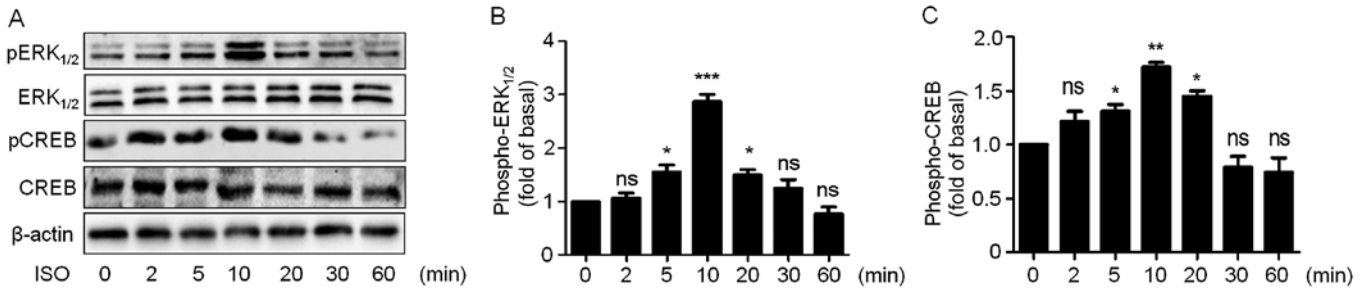

Figure 3. ISO increases $ERK_{1/2}$ and CREB phosphorylation in A549 cells. (A) Cells were cultured in 6-well plates. After starvation with serum-free medium for 24 h, the cells were treated with 10 μM ISO for the indicated times. Cell lysates were subjected to immunoblotting with antibodies against phospho-$ERK_{1/2}$, $ERK_{1/2}$, phospho-CREB, CREB and β-actin. The blots shown are representative of three independent experiments. (B and C) Quantitation of the western blotting results as shown in (A). Data from at least three independent experiments are expressed as means ± SEM vs. control. *P<0.05, **P<0.01, ***P<0.001, ns vs. control. ISO, isoproterenol; $ERK_{1/2}$, extracellular signal-regulated kinase 1/2; CREB, cyclic adenosine monophosphate response element-binding protein; ns, not significant.

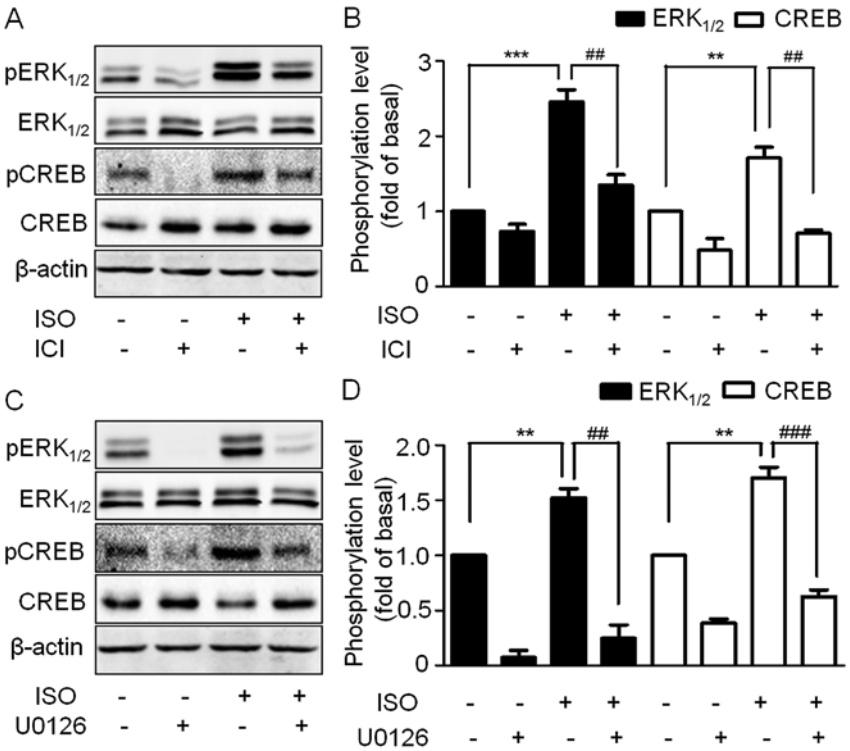

Figure 4. ISO-induced CREB activation is mediated by $ERK_{1/2}$ phosphorylation. (A) Effects of ICI on ISO-induced $ERK_{1/2}$ and CREB phosphorylation. (B) Quantitation of western blotting results as shown in (A). (C) Effects of $MEK_{1/2}$ inhibitor U0126 on ISO-induced $ERK_{1/2}$ and CREB phosphorylation. (D) Quantitation of western blotting results as shown in (C). The blots shown are representative of three separate experiments. Data from at least three independent experiments are expressed as means ± SEM. **P<0.01, ***P<0.001 vs. control, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. ISO. ISO, isoproterenol; CREB, cyclic adenosine monophosphate response element-binding protein; $ERK_{1/2}$, extracellular signal-regulated kinase 1/2; ICI, ICI-118,551.

β_2-AR activation induces $ERK_{1/2}$ and CREB phosphorylation in A549 cells. We demonstrated that ISO significantly increased A549 cell growth. MAPK pathways constitute a large modular network that regulates a variety of physiological processes, such as cell growth, differentiation, and apoptotic cell death. To this end, we firstly monitored $ERK_{1/2}$ phosphorylation levels following ISO treatment. ISO caused a rapid and transient increase in $ERK_{1/2}$ phosphorylation with no changes in $ERK_{1/2}$ expression levels (Fig. 3A and B). $ERK_{1/2}$ phosphorylation peaked at 10 min and then decreased. Similarly CREB, an important transcription factor that plays important roles in cell proliferation, was also phosphorylated in a transient manner and also peaked at 10 min (Fig. 3A and C).

To further confirm the $ERK_{1/2}$ and CREB phosphorylation mediated by β_2-AR, the A549 cells were pre-treated with ICI and then stimulated by ISO. $ERK_{1/2}$ and CREB phosphorylation was blocked by ICI (Fig. 4A and B). As CREB is an important downstream target of $ERK_{1/2}$, we therefore tested whether ISO-induced CREB phosphorylation is mediated through $ERK_{1/2}$. We treated A549 cells with the selective $MEK_{1/2}$ inhibitor U0126. Consistent with our hypothesis, inhibition of the MAPK pathway led to a strong inhibition of ISO-induced $ERK_{1/2}$ and CREB phosphorylation (Fig. 4C and D), thus

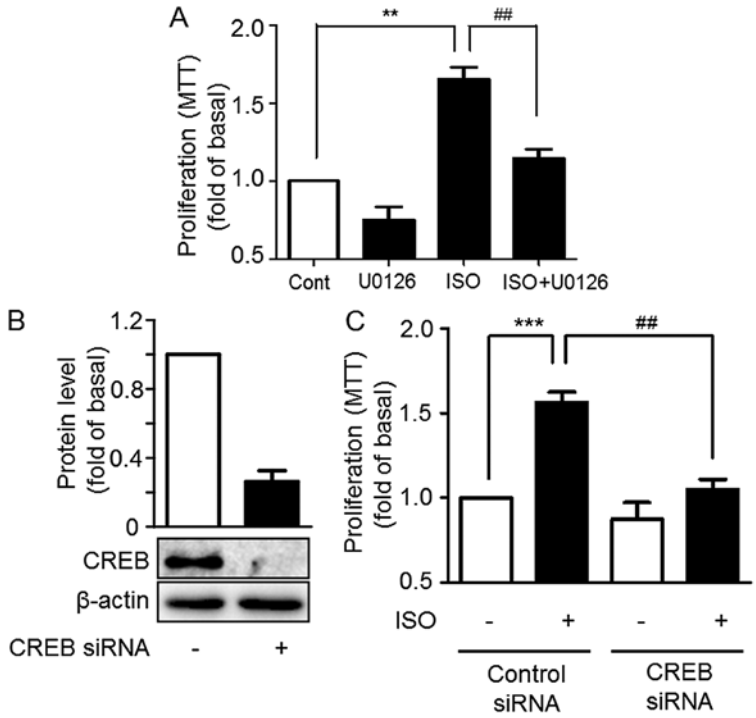

Figure 5. $ERK_{1/2}$ and CREB are required for ISO-mediated proliferation of A549 cells. (A) Effects of $MEK_{1/2}$ inhibitor U0126 on ISO-induced proliferation in A549 cells. The cells were incubated with 10 μM ISO in the absence or presence of U0126 (10 μM). **P<0.01 vs. control, $^{\#\#}$P<0.01 vs. ISO. (B and C) Effects of siRNA against CREB on ISO-induced cell proliferation. Cells were pre-treated with control RNAi or RNAi of CREB for 48 h and then treated with 10 μM ISO. Data are shown as means ± SEM from at least three triplicate experiments. ***P<0.001 vs. basal with control siRNA, $^{\#\#}$P<0.01 vs. ISO-treated cells transfected with control siRNA. $ERK_{1/2}$, extracellular signal-regulated kinase 1/2; CREB, cyclic adenosine monophosphate response element-binding protein; ISO, isoproterenol.

suggesting that the activation of β_2-AR by ISO induces $ERK_{1/2}$ phosphorylation which in turn activates CREB.

$MEK_{1/2}$ and CREB inhibition suppresses ISO-mediated proliferation in A549 cells. Given the ability of ISO to significantly promote the proliferation of A549 cells and induce $ERK_{1/2}$ and CREB phosphorylation, in order to further illustrate whether ISO-mediated $ERK_{1/2}$ and CREB activation are involved in cell proliferation, A549 cells were pre-treated with U0126, and then treated with 10 μM ISO. The proliferative effects of ISO were suppressed by U0126 (Fig. 5A). Furthermore, knockdown of CREB expression by specific siRNA significantly suppressed the A549 cell proliferation in comparison with control siRNA (Fig. 5B and C). All the above results indicate that $ERK_{1/2}$ is involved in ISO-mediated proliferation through CREB phosphorylation in A549 cells.

Knockdown of CREB inhibits ISO-mediated increases in MMP-2, MMP-9 and VEGF levels in A549 cells. MMPs, the gelatinases MMP-2 and MMP-9 in particular, and VEGF have been documented as contributing to the aggressiveness of highly metastatic tumors (14). We then examined the effect of ISO on the expression of MMP-2, MMP-9 and VEGF in the A549 cells. The RT-qPCR results showed that expression levels of the MMP-2, MMP-9 and VEGF genes were significantly upregulated after treatment with 10 μM ISO for 48 h (Fig. 6A). Interestingly, these effects were blocked by knockdown of CREB before ISO treatment (Fig. 6B-D), indicating that the action of ISO on A549 cell invasiveness is through β_2-AR-mediated CREB phosphorylation.

Discussion

Neurotransmitters are signaling substances that traditionally play important roles in both the central and peripheral nervous systems. However, accumulating studies have demonstrated that the stress neurotransmitters adrenaline and noradrenaline have a direct influence on the migration and invasiveness of multiple tumor cells, including cancers of the lung, prostate, colon, stomach, breast and ovary (15-19). Although several studies have preliminarily investigated the role of β-ARs in lung cancer cell lines such as human lung adenocarcinoma cell line NCI-H322 and human peripheral airway cell line HPLD1 (11), the function of β-ARs especially β_2-AR on human lung epithelial-derived A549 cells and the underlying mechanisms have not been well studied. In the present study, we investigated the molecular mechanisms of β_2-AR involved in lung cancer cell proliferation. Our results showed that activation of the β_2-AR by ISO significantly enhanced the proliferation of A549 cells. These effects were found to be mediated by the MAPK/CREB pathway as blocking the MAPK pathway or knockdown of CREB expression was able to suppress ISO-induced A549 cell proliferation.

β-ARs are constitutively expressed in most mammalian cells. However, the distribution and the expression level of these subtypes may vary from tissue to tissue or even from species to species in a given tissue (20). A much higher level of β_2-AR mRNA was detected in hepatocellular carcinoma (HCC) tumor cells, while β_1-AR mRNA was almost undetectable (21,22). Moreover, the β_2-AR density in HCC cellular membranes was much higher than the β_2-AR density

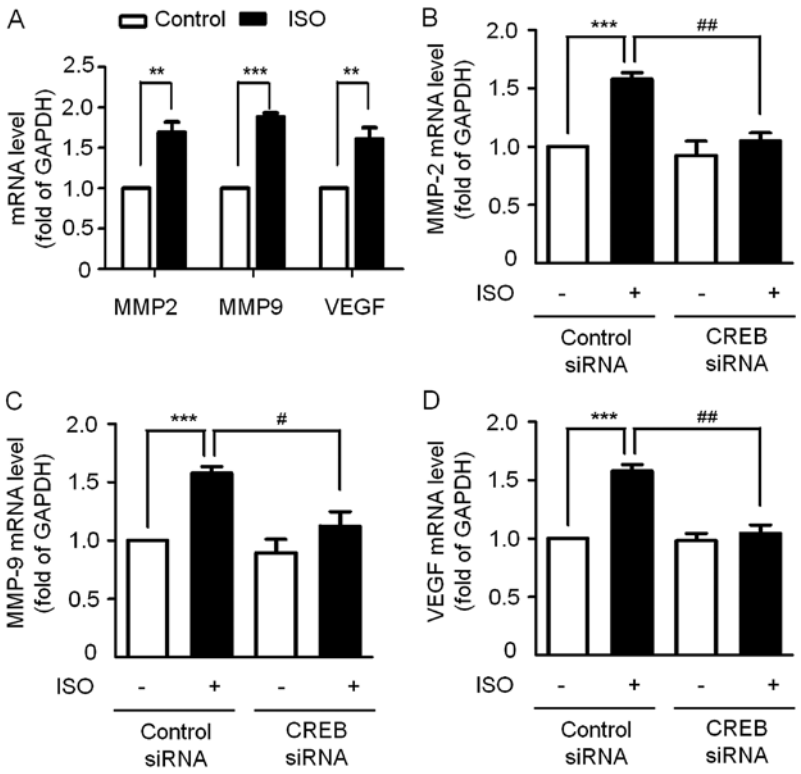

Figure 6. Effects of ISO on the expression of MMP-2, MMP-9, VEGF at the mRNA level. (A) Cells were treated with 10 μM ISO for 48 h, RT-qPCR was performed to detect the mRNA level of MMP-2, MMP-9, VEGF. **P<0.01, ***P<0.001 vs. control. (B-D) Effects of siRNA against CREB on ISO-induced MMP-2, MMP-9, VEGF expression. Data are shown as means ± SEM of three triplicate experiments. ***P<0.001 vs. basal with control siRNA. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. ISO-treated cells transfected with control siRNA. ISO, isoproterenol; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; RT-qPCR, quantitative real-time polymerase chain reaction; CREB, cyclic adenosine monophosphate response element-binding protein.

in non-adjacent non-tumor liver cell membranes (23). β_1-ARs predominated over β_2-ARs in NCI-H322 and NCI-H441 cell lines derived from a human pulmonary adenocarcinoma with Clara cell phenotype (11,24). In the present study, both reverse transcription PCR and RT-qPCR results revealed that the expression level of β_2-ARs was much higher as compared to β_1-ARs in the A549 lung cancer cells. The presence of mRNA does not ensure that the protein is present in the cells in some cases. Nevertheless, none of the commercial antibodies targeting β-ARs work (25) and even much of what is known by using these antibodies has been thrown into doubt (26). However, this has been confirmed by the following functional assay showing that ISO treatment can promote A549 cell proliferation. Moreover, this effect can be completely blocked by ICI, a selective antagonist of β_2-AR. Thus, this confirmed that β_2-ARs are the predominant β-ARs in A549 cells.

As demonstrated in several studies, agonists binding to β-ARs activate the heterotrimeric Gαs proteins to stimulate adenylyl cyclase synthesis of cAMP. The transient cAMP flux can initiate multiple cellular processes via two major downstream effector systems including cAMP-dependent protein kinase (PKA) and guanine nucleotide exchange protein activated by adenylyl cyclase (EPAC) (8), both of which play important roles in cell morphology and motility. Previous studies suggest that β_2-AR exerts an effect on carcinogenesis mainly through activating signaling via adenylyl cyclase and its downstream effectors cAMP, PKA, CREB and STAT3 as well as transactivation of the EGFR pathway. In NCI-H322 and HPLD1 lung cancer cell lines, NNK, an agonist for β-ARs, has been shown to upregulate $ERK_{1/2}$ and CREB/ATF-1 phosphorylation (11). However, it is not known whether they are also the effectors downstream of β_2-AR in A549 cells. In our study, we found that ISO treatment significantly induced $ERK_{1/2}$ and CREB phosphorylation. The $MEK_{1/2}$ inhibitor U0126 used to block $ERK_{1/2}$ activity inhibited CREB activation. However, whether the EGFR transactivation pathway was involved in this process was not investigated. Moreover, in addition to heterotrimeric G proteins, more and more biochemical and cellular studies indicate that β_2-ARs may induce $ERK_{1/2}$ activation through a wide variety of intracellular proteins that are independent of heterotrimeric G protein activation (27). Among these intracellular proteins, β-arrestins and Src have been shown to initiate a broad variety of signaling events such as the activatation of $ERK_{1/2}$ signaling upon β_2-AR activation (28). These are also the possible candidate proteins that may be involved in β_2-AR-induced $ERK_{1/2}$ activaton in A549 cells.

The ability of tumor cells to invade the extracellular matrix plays an important role in invasion and metastasis. MMPs are key factors in the degradation of the components of the extracellular matrix and therefore have been regarded as major critical molecules assisting tumor cells during metastasis (29-31). Studies have shown that the catecholamine hormones may influence cancer progression by modulating the expression of MMPs and VEGF in ovarian cancer cells (19,32). In the present study, we found that ISO had a role in modulating the expression of MMP-2, MMP-9 and VEGF in the A549 cells, which may contribute to the aggressiveness

of the highly metastatic types of lung cancer cells. Notably, the ISO-mediated upregulation of MMP-2, MMP-9 and VEGF was inhibited by the knockdown of CREB expression.

Acknowledgements

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