이학박사 학위논문

Molecular mechanism for cAMP signaling to regulate HDAC6 expression in non-small cell lung cancer

cAMP 신호전달계가 폐암세포주에서 HDAC6의 발현을 조절하는 분자기전

2016년 2월

서울대학교 대학원

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Abstract

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Stress conditions are correlated with tumor growth, progression and metastasis. Epinephrine signaling is activated during stress conditions. Isoproterenol, an epinephrine analog, binds and activates β-adrenergic receptor to stimulate cAMP production and regulate various cellular metabolism and gene expression. α-Tubulin is deacetylated by histone deacetylase 6 (HDAC6), which affects the microtubule dynamics to regulate cell migration. This study investigated the mechanism for stress signals to modulate the migration of non-small cell lung cancer cells via regulation of HDAC6 expression. Treatment of H1299 lung cancer cells with isoproterenol decreased the acetylation level of α-tubulin and stimulated cell migration. Isoproterenol treatment also increased the expression of HDAC6. Isoproterenol-stimulated cell migration was blocked by

knockdown of HDAC6. Treatment with N6-phenyl-cAMP, a selective activator of protein kinase A (PKA), and 8-pCPT-2'-O-Me-cAMP (8-pCPT), a selective activator of exchange protein activated by cAMP (Epac), increased HDAC6 expression. Isoproterenol and 8-pCPT increased Rap1 activity, which acts downstream of Epac. Constitutively active Rap1A increased HDAC6 expression and the knockdown of Rap1A decreased isoproterenol-induced HDAC6 expression. Isoproterenol inhibited External signal-activated kinase (ERK) in H1299 cells. Inhibition of ERK increased HDAC6 levels, and the expression of constitutively active MAPK kinase (MEK1) decreased isoproterenol-induced HDAC6 expression. It is concluded that isoproterenol increases HDAC6 expression via PKA/Epac/ERK-dependent pathway, and isoproterenol-induced HDAC6 expression increases the migration of lung cancer cells. This study suggests that stress signal can stimulate the migration of cancer cells by increasing the expression of HDAC6.

Keywords: cAMP; cell migration; histone deacetylase 6; isoproterenol; lung cancer; stress

Student Number : 2011-30621

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Introduction

1. The stress response and its associated signaling pathways

Stress affects the immune system, increases the production of pro-inflammatory cytokines, and also promotes depression, anxiety and drug-seeking behavior (1). It can influence the response to infectious disease, inflammation, and aging (2). Stress causes a fight-or-flight response, inducing the production of catecholamine neurotransmitters such as epinephrine and norepinephrine by the sympathetic nervous system and adrenal medulla (3, 4). Epinephrine binding to β-adrenergic receptor activates Gs protein (5), which activates adenylyl cyclase and increases the intracellular concentration of 3′, 5′-cyclic adenosine monophosphate (cAMP). An elevated concentration of cAMP regulates cellular functions including metabolism, differentiation, gene expression, and migration. cAMP regulates the protein kinase

A (PKA) and exchange protein directly activated by cAMP (Epac) pathways (6). PKA phosphorylates cAMP response element binding protein (CREB) (7, 8) and phosphorylated CREB promotes many kinds of target gene activation. Epac binds and activates a downstream effector, the Ras-like small GTPase Rap, which regulates cell adhesion, proliferation, and apoptosis (9).

2. G protein

2.1 Heterotrimeric G protein

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are composed of 3 subunits (α, β and γ) and exist in almost all cells. They induce signaling responses and regulate various biological phenomena such as cell growth, differentiation, and metabolism. When unstimulated, the G protein is bound to guanine diphosphate (GDP), but upon activation by an extracellular ligand, GDP is exchanged for guanine triphosphate (GTP), resulting in the G protein undergoing a conformational change to its active form (10).

When GTP binds to the Gα subunit, the Gβγ s subunit dissociates from Gα (11). The α subunit of G proteins each consist of 390-395 amino acids, and they are divided into 4 classes: Gs, Gi, Gq, and G12 (12). Gs stimulates adenylyl cyclase and regulates Ca^{2+} channels. Gi inhibits adenylyl cyclase, regulates K^{+} and Ca^{2+} channels, and activates cyclic guanine monophosphate (cGMP) phosphodiesterase. Gq activates phospholipase C (PLC), and G12 regulates Na^{+}/K^{+} exchange (13). The β subunit has five different subunits and γ has 12 different subunits. Gβγ dimers activate effectors including PLCβ2, β3, β-adrenergic receptor kinase and phosphoinositide 3-kinase (PI3 kinase) (14).

2.2 3', 5'-cyclic adenosine monophosphate (cAMP)

3′, 5′-cyclic adenosine monophosphate (cAMP) was first identified as a ubiquitous second messenger regulated by adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE) (15, 16). When a ligand binds to a G protein coupled receptor (GPCR), it activates AC to

produce cAMP from ATP. Intracellular cAMP regulates various cellular functions including metabolism, differentiation, gene expression, cell growth and division, apoptosis, and migration. cAMP regulates several effectors, including PKA, Epac, and the cyclic nucleotide-gated ion channels (Figure 1) (6, 17, 18). cAMP also undergoes cross-talk with many intracellular signaling molecules.

2.3 cAMP-dependent protein kinase A (PKA)

Inactive PKA consists of two catalytic subunits and two regulatory subunits. Two classes of regulatory subunits (RI and RII) and three isoforms of catalytic subunits (Cα, Cβ and Cγ) have been identified. When cAMP binds to the regulatory subunits of PKA to activate PKA signaling, the regulatory subunits undergo conformational changes and are released from the catalytic subunits, which then phosphorylate their substrates: cAMP response element binding protein (CREB), cAMP response element binding modulator (CREM), and activating transcription factor 1 (ATF1). The phosphorylation

of these substrates is essential for them to interact with the transcriptional coactivators, CREB-binding protein (CBP) and p300 (15, 19).

2.4 Exchange protein directly activated by cAMP (Epac)

Epac binds and activates downstream effectors including the Ras-like small GTPases Rap1 and Rap2 to regulate cell adhesion, proliferation, and apoptosis (9). Epac has two isoforms, Epac1 and Epac2, which share a high degree of sequence homology and both have a regulatory region at the N-terminus and a catalytic region at the C-terminus (6). The expression of Epacs has been verified in many cell types. Epac1 is highly expressed in the thyroid, kidney, ovary, and skeletal muscle. Epac2 is mostly expressed in the brain and adrenal gland (20). Downstream of Epacs, Rap1 is regulated by many cellular processes and activated by intracellular molecules including cAMP (21). PKA also activates Rap1, but in a cAMP-independent manner (6, 22).

3. Epigenetics and histone modification

Gene expression is usually regulated by how tightly DNA is packaged in eukaryotic cells. The nucleosome, a chromatin subunit, is composed of an octamer of 4 core histones, comprising 2 H2A/H2B dimers and an H3/H4 tetramer. DNA is surrounded by this nucleosome. In resting cells, DNA is compacted to prevent binding of transcription factors; however, this compact structure can be partly unpacked via posttranslational modification of the histone proteins, allowing transcription factors to bind to the DNA and initiate gene transcription (23).

Histone modifications include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, adenosine diphosphate–ribosylation, deimination, and proline isomerization. Many kinds of enzymes are involved in regulating each type of modification. Among these modifications, acetylation is the best understood (24).

3.1 Histone acetyltransferase (HAT)

Histone acetylation is a reversible process and it regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs are divided into three families; Gcn5-related N-acetyltransferase, MYST, and CBP/p300. Acetylation at the H3 K56 residue was recently reported. The K56 residue is located in the major groove of the DNA so it may be involved in mediating direct interactions between DNA and H3 (24, 25).

3.2 Histone deacetylase (HDAC)

Histone deacetylases (HDACs) are divided into two families; classical HDACs and NAD^+-dependent HDACs SIR2 family. . Classical HDACs are divided into three subclasses based on their homology with yeast proteins. The class I HDACs (HDAC1, 2, 3, and 8) are usually localized in the nucleus. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) are predominantly localized in the cytoplasm and can translocate to the nucleus (23) (Figure 2). HDAC11 is in class IV, which

has recently been identified (26).

3.3 Histone deacetylase 6 (HDAC6)

Histone deacetylase 6 (HDAC6) belongs to HDAC class IIb and is the best characterized of the HDACs that have 2 tandem deacetylase domains. HDAC6 is predominantly localized in the cytoplasm because it has a nuclear export domain at the N-terminus and a SE14 domain at the C-terminus (27). Also, there is a zinc finger ubiquitin binding domain at the C-terminus of HDAC6, which binds with ubiquitin to regulate misfolded proteins (Figure 3) (28). Unlike other HDACs, HDAC6 deacetylates non-histone proteins, such as α-tubulin, Hsp90, cortactin, and Ku70. Therefore, it regulates many important biological processes including cell migration, inflammation, proliferation, and apoptosis (Figure 4). α-tubulin was the first identified substrate of HDAC6 and the acetylation of α-tubulin regulates microtubule dynamics to promote cell migration (29).

4. Mitogen-activated protein kinases (MAPKs)

MAPKs are serine- and threonine-specific kinases that regulate many cellular functions including gene expression, cell cycle control, and apoptosis. MAPKs are activated by a classical cascade of consecutive phosphorylation events. MAPK kinase kinases (MAPKKKs) phosphorylate MAPK kinases (MAPKKs), which phosphorylate and activate MAPKs. There are three MAPK subfamilies: the extracellular signal-related kinases (ERK1 and 2); the c-Jun N-terminal kinases (JNK1, 2, and 3); and the p38 enzymes (p38α, p38β, p38γ and p38δ)(Figure 5) (30).

4.1 Extracellular signal-related kinases (ERKs)

ERKs are widely expressed and regulate many cellular functions including gene transcription, proliferation, and differentiation. Various stimuli can activate the ERK pathway, such as cytokines, growth factors, and GPCR activators. The small GTP-binding protein Ras is an upstream regulator of ERK activation. Ras phosphorylates and activates a MAPKKK

(Raf), which phosphorylates a MAPKK (MEK), which phosphorylates and activates ERK. The ERK signaling pathway regulates proliferation through downstream targets including NF-kB, Ets-1, AP-1 and c-Myc. The ERK signaling pathway is abnormally activated in malignantly transformed cells and ERK inhibitors are used in cancer therapy. ERK signaling interacts with the phosphoinositide 3-kinase/Akt pathway to regulate the balance between cellular proliferation and apoptosis (30, 31). The ERK pathway and its interactors include important therapeutic targets.

5. Tumor cell migration, invasion and metastasis

Metastasis is an important process for cancers and causes 90% of the deaths ascribed to solid tumors (32). Tumor metastasis occurs through a complicated and multistep process. Understanding the mechanisms and methods for the inhibition of metastasis are essential for improving cancer therapy. Cancer cell migration is an initial step of invasion and tumor metastasis. The general mechanism of migration is

divided into 4 steps: protrusion, adhesion, contraction, and retraction. Cell migration occurs via a microtubule-dependent and actin-dependent pathway. Microtubules regulate cell adhesion to the extracellular matrix and the disassembly of focal adhesions (33). The stabilization of microtubules could be associated with the posttranslational modification of tubulin. Acetylated tubulin is observed in stable microtubules and its acetylation is completely reduced in the fibroblast leading edge, which is involved in cell movement (29). In the actin-dependent pathway, there are key regulatory proteins including Wiskott-Aldrich syndrome protein family proteins, Arp2/3 complex, LIM-kinase, cofilin and cortactin (34).

6. Stress and HDACs

Stress signaling involves HDACs. HDAC1 was reported to be involved in the regulation of kappa-opioid receptor (KOR) expression by behavioral stress (1), chronic social stress was associated with decreased levels of HDAC2 in the nucleus accumbens in mice (35), and HDAC6 inhibition

was found to induce proresilience and antidepressant interventions (36). In addition, HDAC6 regulates glucocorticoid receptor chaperone, which is activated under conditions of psychosocial stress (37). Therefore, stress signaling might affect tumor growth and progression by the epigenetic control of gene expression.

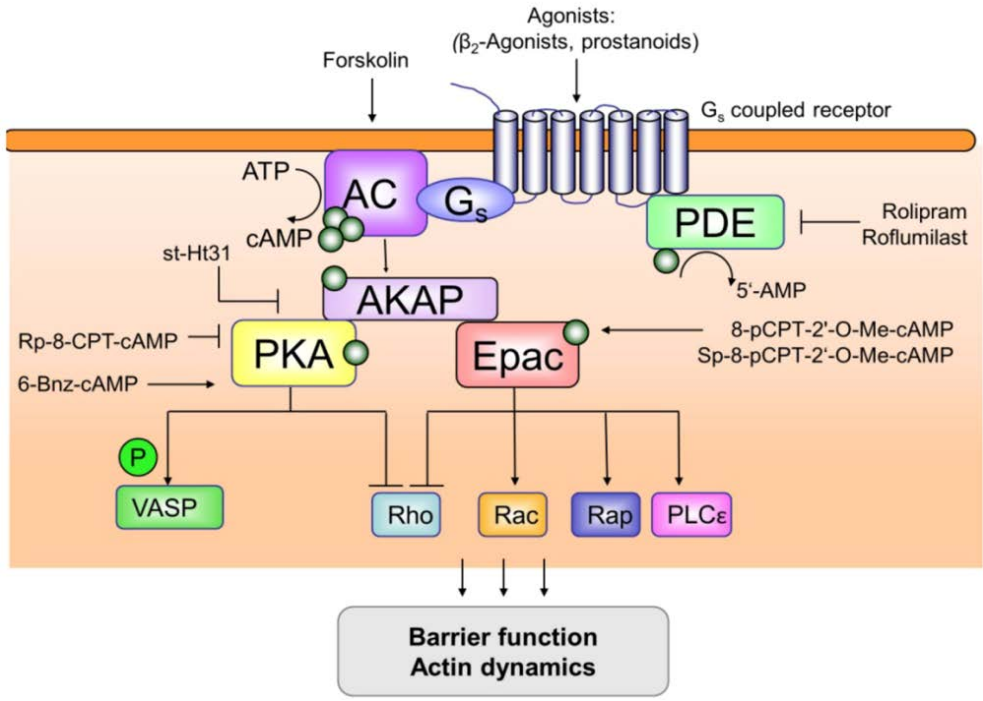


Figure 1. cAMP signaling pathway (17)

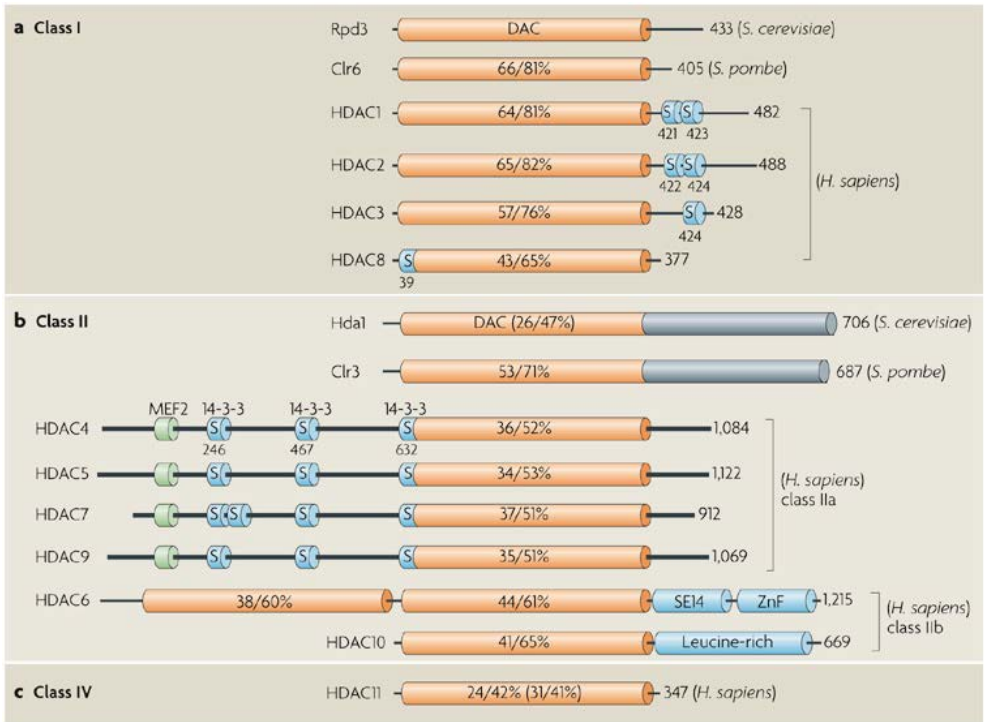


Figure 2. Domain of classical HDAC families (38)

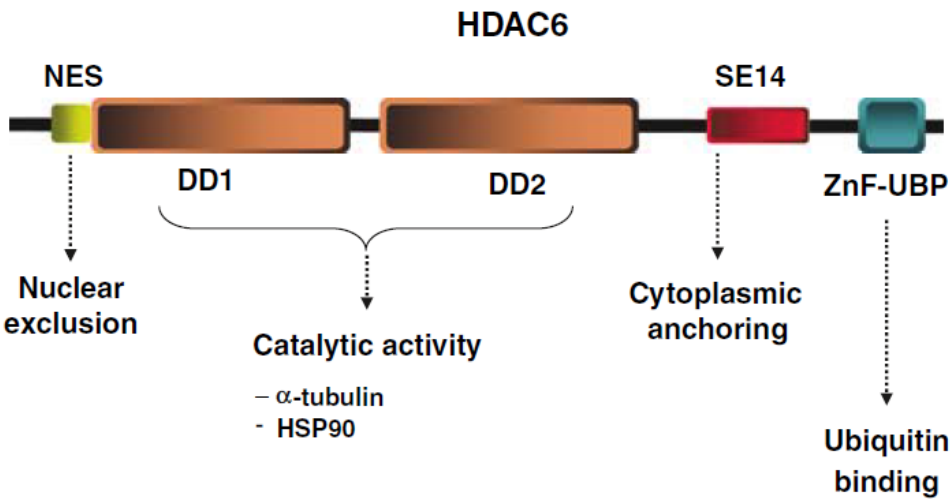


Figure 3. Functional domains of HDAC6 (39)

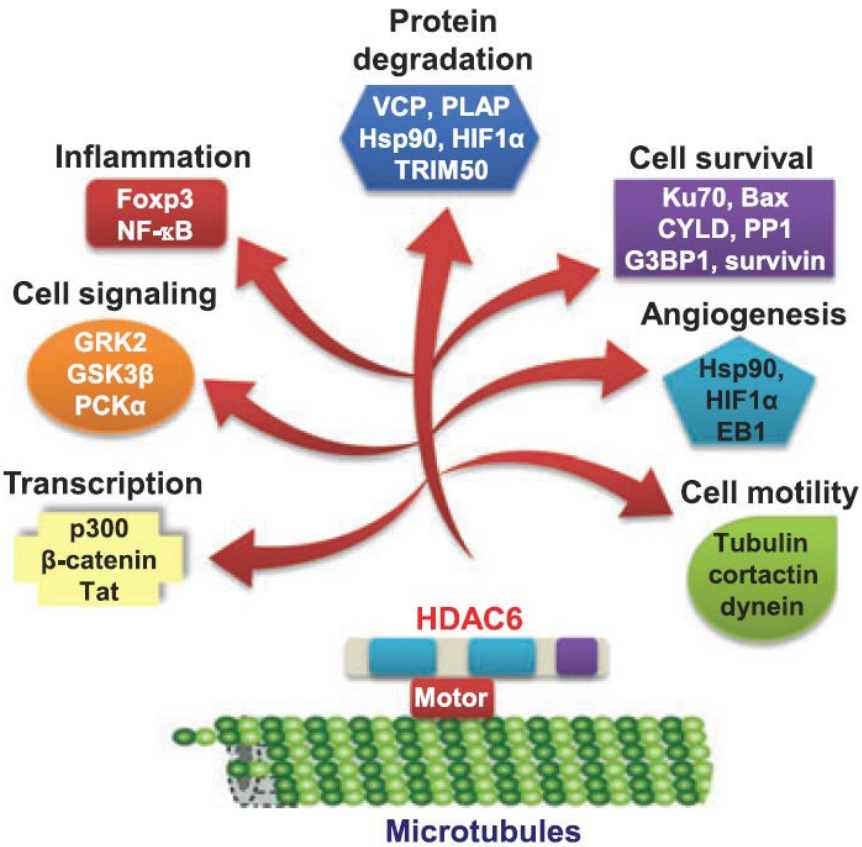


Figure 4. HDAC6 substrates and their functions (27)

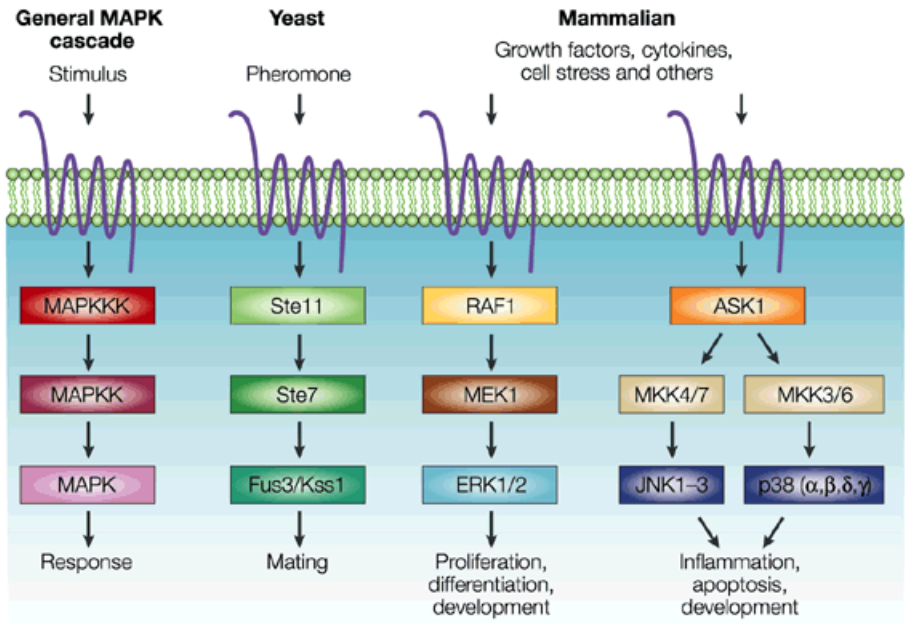


Figure 5. Mitogen-activated protein kinase (MAPK) cascades (40)

Purpose

This study aimed to investigate the effect of stress signal on the cell migration of non-small cell lung cancer cells and its underlying mechanisms.

The specific aim of this study was:

1. To investigate the effect of isoproterenol on the migration of lung cancer cells
2. To investigate the mechanism of isoproterenol to stimulate migration of lung cancer cells
3. To investigate the mechanism of isoproterenol to increase the expression of HDAC6 in lung cancer cells

Materials and Methods

1. Cell culture and reagents

The human non-small cell lung cancer H1299 and A549 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). These cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI 1640 containing 10% fetal bovine serum (FBS) (Welgene, Taegu, Korea) and 100 units/ml penicillin/streptomycin. The cells were cultured in a 5% CO_2 incubator at 37°C.

Forskolin and SP600125 were purchased from Calbiochem (CA, USA). SB203580 was purchased from Cayman (MI, USA). Actinomycin D, dimethyl sulfoxide (DMSO), Dulbecco's phosphate-buffered saline (DPBS), H89, isoproterenol (ISO), PD98056, PD0325901 and prostaglandin E_2 (PGE_2) were purchased from Sigma Chemicals (MO, USA). N6-phenyl-cAMP (6-Phe-cAMP) and 8- (4-cholorophenylthio)-2'-O-methyl-cAMP (8-pCPT-cAMP) were purchased from the Biolog Life Science Institute (Bremen, Germany).

2. Expression plasmids and transient transfection

The expression plasmid of the EE-tagged constitutively active mutant of Gαs (GαsQ227L) was purchased from the Missouri S&T cDNA Resource Center (MO, USA). The GαsQ277L mutant has a substitution of Leu-277 for Gln-277 that results in the inactivation of the intrinsic GTPase, which causes constitutive activation of the protein. Wild-type and dominant-negative CREBs (S133A, R287L) were gifts from Dr. Sahng-June Kwak (Dankook University, Cheonan, Korea). CARap1A/1B was kindly provided by Keith Burridge (University of North Carolina, USA) (41), the dominant negative PKA in MT-REVab was provided by Dr. G. Stanley McKnight (University of Washington, USA), and the catalytic subunit of PKA (GPKA) was provided by Dr. S.H. Green (42). The short hairpin RNAs (shRNAs) against HDAC6 and Rap1 and the control shRNA were purchased from Sigma Chemicals. Small interfering RNAs (siRNAs) against Epac1 and Epac2 were from Santa Cruz Biotechnology (CA, USA). Decoy oligonucleotides for the CRE (CRE decoy) were prepared as described previously (43). The H1299 cells were transfected with expression constructs and siRNAs using Lipofectamine 2000 (Invitrogen, CA, USA).

3. Quantitative reverse transcription polymerase chain reaction (qPCR)

The total RNA was isolated from the cells using the TRIzol reagent (Invitrogen). First-strand complementary DNA (cDNA) was synthesized using oligo-dT primers and the SuperScript II Reverse Transcriptase (Invitrogen). The specific oligonucleotide primers used in the qPCR were as follows: for HDAC6, 5'-TCAGGTCTACTGTGGTCGTT-3' and 5'-TCTTCACATCTAGGAGAGCC-3'; and for glyceraldehyde-3-phosphase dehydrogenase (GAPDH), 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'.

The qPCR was performed in a 20-μl mixture composed of forward and reverse primers (5 pmol) and SYBR Premix Ex Taq (Takara Bio Inc., Japan) using a C1000 thermal cycler (Bio-Rad, CA, USA). After 40 cycles of PCR, the average threshold cycle (Ct) values from triplicate qPCR experiments were normalized against the average Ct value of GAPDH.

4. Western blot analysis

The expressions of the proteins were analyzed by western blotting using specific antibodies. Antibodies against HDAC6 and Epac were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against HDAC4, acetylated tubulin, tubulin, and β-actin were from Sigma Chemicals. Antibodies against HDAC7, cAMP response element binding protein (CREB), phospho-CREB (Ser-133), c-Raf, phospho-c-Raf at Ser-338 and Ser-259, MEK1/2, phospho-MEK1/2, p44/42 MAPK, phospho-p44/42 MAPK, and Rap1A/1B were purchased from Cell Signaling Technology (MA, USA). The HDAC9 antibody was from Abcam (Cambridge, UK). The HDAC5 antibody was from Alexis Biochemicals (PA, USA). The horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Zymed (CA, USA) or Santa Cruz Biotechnology. The blots were incubated with an enhanced chemiluminescence substrate mixture (Pierce, Chester, UK), and the resulting blot images were then recorded with an LAS-3000 luminescent image analyzer system (Fuji, Tokyo, Japan). The densities of the protein bands were quantified using the Multi Gauge v3.0 software (Fuji), and the protein amounts are expressed as

multiples of the corresponding densities of the control.

5. Transwell migration assay

H1299 cells were transfected with shRNA targeting HDAC6 or scrambled sequences and incubated for 48 h. The cells (10,000) were then seeded onto the upper chamber (8-μm pore size) of a 24-well plate (Corning, Lowell, USA) containing DMEM, 1% FBS, and 20 μM isoproterenol. The lower chamber was filled with DMEM containing 10% FBS and 20 μM isoproterenol. After incubation for 16 h, the migrated cells were fixed and stained using Diff-Quik solution (Sysmex, Kobe, Japan), and the non-migrated cells were removed with a cotton swab. The cell numbers were counted in five different microscopic fields (Leica DFL 290, Wetzlar, Germany).

6. Assay of Rap1 activity

Rap1 activity was assayed by analyzing the binding of the activated proteins to the Rap1 binding domain (RBD) of the ral guanine nucleotide exchange factor (RalGDS) protein. The plasmid

encoding a GST fusion protein containing the 97-amino acid RBD of the RalGDS protein (pGEX RalGDS-RA) was a gift from J.H. Chung (NIH, USA)) (44). The cell lysates were incubated with GST-RalGDS RBD protein that was preincubated with glutathione-Sepharose 4B (GE Healthcare, USA) at 4 °C with agitation. The beads were washed three times with lysis buffer and then subjected to immunoblotting analysis.

7. Data analysis

All experiments were independently repeated at least three times, and the data are presented as the means ± the standard errors (SE). The non-parametric Mann-Whitney U-test was used to analyze the mean values, and P-values less than 0.05 were considered statistically significant.

Results

1. Isoproterenol signaling increases the expressions of HDAC6 protein and mRNA in H1299 lung cancer cells.

To examine the effects of stress signals on the expressions of HDACs, we treated H1299 human lung cancer cells with isoproterenol, which is an analog of the epinephrine stress hormone, and the expressions of HDACII family proteins were analyzed by western blotting. Treatment with isoproterenol increased the expression of HDAC6 without significantly altering the expressions of the other tested HDACII proteins ($p < 0.05$; Figure 6). Furthermore, treatment of the H1299 cells with another Gαs-coupled receptor agonist (prostaglandin E_2) or an adenylyl cyclase activator (forskolin) also increased HDAC6 expression (Figure 7). When the cells were treated with isoproterenol, the expression of HDAC6 mRNA increased at 6 h (Figure 8) and reached a plateau at 30 h, and the expression of the HDAC6 protein began to increase at 12 h (Figure 9). Dose dependent treatment manner of isoproterenol, 1 μM

to 100 μM of isoproterenol increased HDAC6 expression (Figure 10). To examine whether increased transcription might have caused the increase in HDAC6 expression, the effect of transcription inhibition via actinomycin D treatment on the isoproterenol-induced HDAC6 expression was assessed. Treatment with actinomycin D abolished the isoproterenol-induced increases in HDAC6 mRNA (Figure 11) and protein expressions (Figure 12). These results indicate that isoproterenol signaling increases HDAC6 protein expression by stimulating the transcription of the HDAC6 gene via cAMP signaling in lung cancer cells.

2. Isoproterenol decreases the acetylation of α-tubulin and increases the migration of H1299 lung cancer cells in an HDAC6-dependent manner.

HDAC6 deacetylates many non-histone proteins, including α-tubulin, and the acetylation of α-tubulin is involved in the regulation of cell migration. Thus, the effects of isoproterenol on the acetylation of α-tubulin and cell migration were examined. Treatment with isoproterenol significantly reduced the acetylation of α-tubulin at 36 h and 48 h after the treatment (Figure 13). When HDAC6 expression

was knocked down via transfection with HDAC6 shRNA, the acetylation of α-tubulin was increased (Figure 14). Treatment with isoproterenol increased the migration of the H1299 cells in the transwell migration assay, and this increase in migration was blocked by knockdown of HDAC6 with a specific shRNA (Figure 15). These results indicate that isoproterenol decreases the acetylation of α-tubulin and promotes cell migration by increasing HDAC6 expression in H1299 lung cancer cells.

3. Isoproterenol increases HDAC6 by inhibiting c-Raf-MEK-ERK pathways.

To probe the signaling pathway that mediated the HDAC6-increasing effect of isoproterenol signaling, the effects of mitogen-activated protein kinases (MAPKs) on HDAC6 expression were assessed following treatment with MAPK-specific inhibitors. Treatment with the extracellular signal-regulated kinase (ERK) inhibitor (PD98059) caused an increase in HDAC6 expression that was similar to that of isoproterenol treatment. However, treatments with a JNK inhibitor (SP600215) and a p38 inhibitor (SB203580) did not significantly alter the HDAC6 expression levels (Figure 16).

Treatment with PD0325901, another ERK inhibitor, also caused a significant increase in HDAC6 expression (Figure 17). Treatment with PD98059 increased the HDAC6 mRNA level, and the inhibition of transcription via actinomycin D abolished the effects of PD98059 on HDAC6 mRNA (Figure 18) and protein (Figure 19). Moreover, treatment with isoproterenol decreased the activating phosphorylation of ERK1/2, the upstream mitogen-activated extracellular signal-regulated kinase (MEK1/2), and c-Raf (S338) from 30 min until 4 h after the treatment (Figure 20). Furthermore, the expression of constitutively active MEK1 (caMEK1) abolished the isoproterenol-induced HDAC6 expression (Figure 21). Additionally, treatment of A549 cells, i.e., another human lung cancer cell line, with forskolin also inhibited the c-Raf-MEK1/2-ERK1/2 activity and increased the HDAC6 expression (Figure 22). These results indicate that isoproterenol increased HDAC6 expression via the inhibition of the c-Raf-MEK1/2-ERK1/2 signaling pathway in lung cancer cells.

4. Both the PKA and Epac pathways mediate isoproterenol-induced inhibitions of c-Raf.

To investigate the mechanism by which isoproterenol inhibited c-Raf-MEK1/2-ERK1/2 to increase HDAC6 expression, we analyzed the roles of the cAMP-dependent protein kinase (PKA) and exchange protein activated by cAMP (Epac) pathways, which are the two major signaling pathways that are activated by cAMP. The expression of a dominant negative PKA (dnPKA) did not block the isoproterenol-induced HDAC6 expression (Figure 23). Similarly, the knockdown of Epac1/2 with specific siRNAs did not block the isoproterenol-induced HDAC6 expression (Figure 24). However, the simultaneous transfection of dnPKA and Epac1/2 siRNAs abolished the isoproterenol-induced HDAC6 expression (Figure 25). The involvements of PKA and Epacs in HDAC6 expression were confirmed in a study utilizing selective agonists. Treatment with the PKA-selective agonist N6-phenyl-cAMP (6-Phe-cAMP) or the Epac selective agonist 8-pCPT-2'-O-Me-cAMP (8-pCPT-cAMP) decreased the activating phosphorylation of c-Raf at Ser-338 and increased the inhibitory phosphorylation of c-Raf at Ser-259. The inhibitions of c-Raf by these agonists resulted in decreased

phosphorylation of the downstream MEK1/2 and ERK1/2, which caused an increase in HDAC6 expression similar to that of isoproterenol treatment (Figure 26). Moreover, the expression of the catalytic subunit of PKA (GPKA) also induced HDAC6 expression (Figure 27). Additionally, the mediation of the 6-Phe-cAMP effect on HDAC6 expression by PKA was confirmed by the abolishment of the 6-Phe-cAMP effect by the expression of a dominant negative PKA (Figure 28). These results indicate that both the PKA and Epac pathways mediated the isoproterenol-induced inhibition of c-Raf and the increase in HDAC6 expression.

5. Rap1A mediates the Epac-induced inhibition of c-Raf.

To study how the Epac pathway inhibits c-Raf, we assessed the role of Rap1, which is a well-known downstream target of Epac. The effect of the Epac selective activator, 8-pCPT-cAMP, on HDAC6 expression was blocked by the knockdown of Epac with siRNA, which confirmed the selectivity of the agonist for Epac (Figure 29). Treatment with 8-pCPT-cAMP or isoproterenol increased the amount of Rap1 bound to Rap1 binding domain of the RalGDS protein in a pull-down assay (Figure 30). Because Rap1 has two isoforms,

i.e., Rap1A and Rap1B, we determined which isoform mediated the HDAC6-expressing effect by transfecting each of the constitutively active forms of Rap1 into the H1299 cells. The H1299 cells that expressed the constitutively active Rap1A (caRap1A) exhibited increased HDAC6 expression and decreased phosphorylations of c-Raf, MEK1/2 and ERK1/2, but the cells that expressed the constitutively active Rap1B (caRap1B) did not exhibited increased HDAC6 expression or decreased phosphorylation of c-Raf (Figure 31). The knockdown of Rap1A with shRNA abolished the 8-pCPT-cAMP-induced HDAC6 expression, which confirmed the mediating role of Rap1A (Figure 32). These results indicate that Epac increased HDAC6 expression by activating Rap1A.

To study whether ERK mediates isoproterenol-induced HDAC6 expression via CREB, a well-known transcription factor that is activated by PKA, the effect of CREB inhibition by transfecting dominant negative CREBs or CRE decoy oligonucleotides on PD98059-induced expression of HDAC6 were analyzed. The PD98059-induced increase in HDAC6 expression was not abolished by the transfection dominant negative CREBs (S133A and R287L) or CRE decoy oligonucleotides (Figure 33 & 34). CREB inhibition by the

dominant negative CREB (S133A) or CRE decoy oligonucleotides also abolished the increase in HDAC6 expression by isoproterenol treatment (Figure 35). These results suggest that isoproterenol increases HDAC6 expression in a CREB-independent pathway.

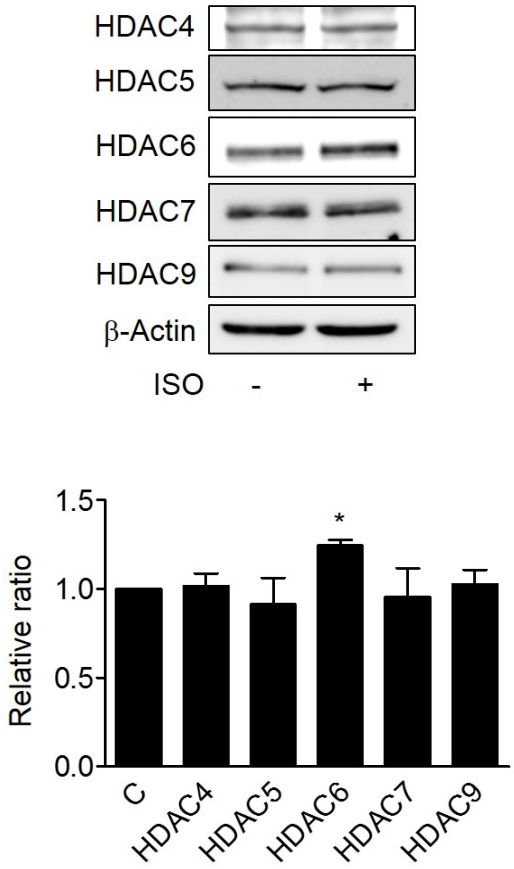


Figure 6. Effects of isoproterenol on the expression of type II HDACs in H1299 human lung cancer cells.

H1299 human lung cancer cells were treated with 20 μM isoproterenol (ISO) and harvested at 48 h for western blot analysis. β-Actin was analyzed as loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

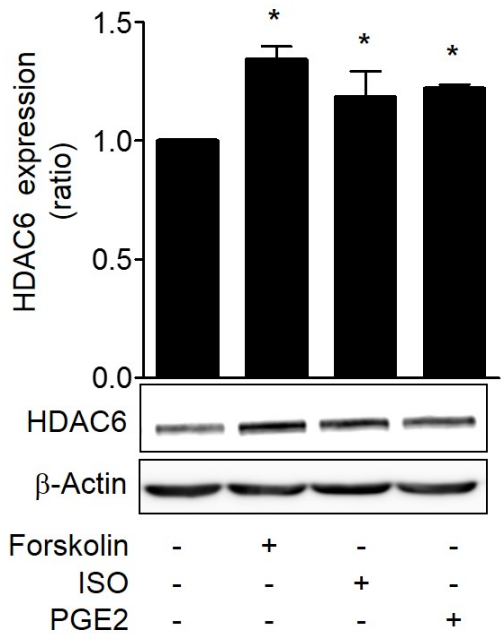


Figure 7. Effects of cAMP signaling on the expression of HDAC6.

H1299 human lung cancer cells were treated with 20 μM isoproterenol (ISO), 20 μM forskolin or 20 μM PGE_2 and harvested at 48 h for western blot analysis. β-Actin was analyzed as loading control. Western band density was quantified using Multi Gauge v.2.3 software and expressed as a ratio relative to the control band density. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

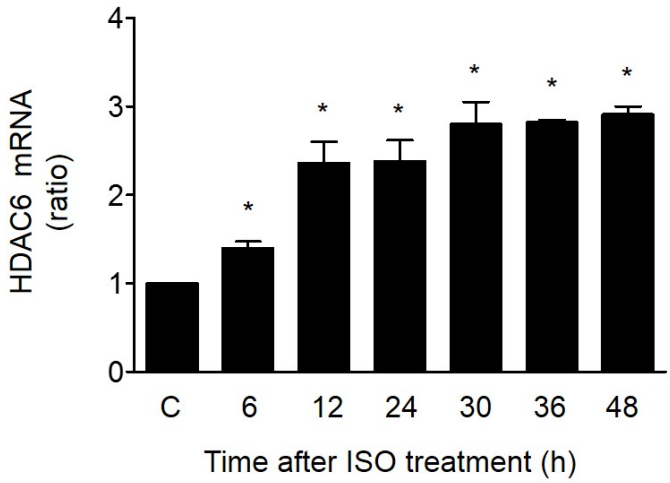


Figure 8. Temporal patterns of the expression of HDAC6 mRNA following isoproterenol treatment.

H1299 cells were treated with 20 μM isoproterenol and harvested at the indicated time points. The HDAC6 mRNA level was analyzed by quantitative real time PCR, normalized to GAPDH mRNA, and expressed as a ratio relative to the control level. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

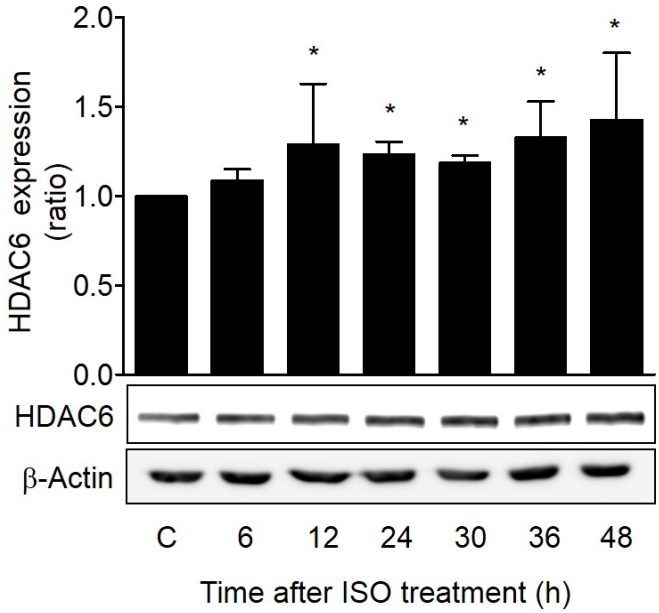


Figure 9. Temporal patterns of the expression of HDAC6 protein following isoproterenol treatment.

H1299 cells were treated with 20 μM isoproterenol and harvested at the indicated time points for western blot. β-Actin was analyzed as a loading control. The western blot band densities were quantified using Multi Gauge v.2.3 software and are expressed as ratio relative to the control band density. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

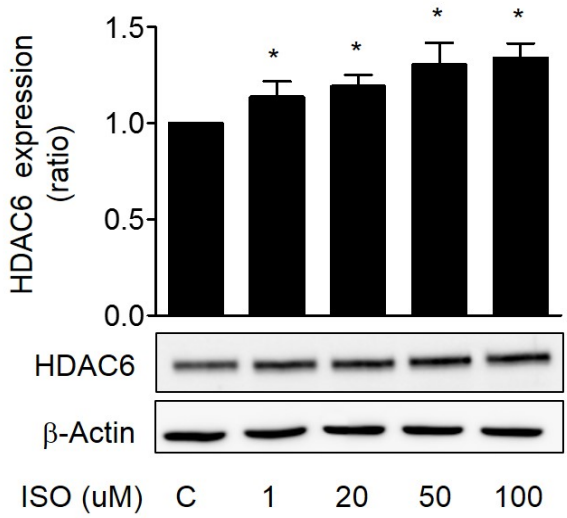


Figure 10. Dose dependent manner of the expression of HDAC6 protein following isoproterenol treatment.

H1299 cells were treated with 1-100 μM isoproterenol and harvested at 48 h for western blot analysis. β-Actin was analyzed as loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

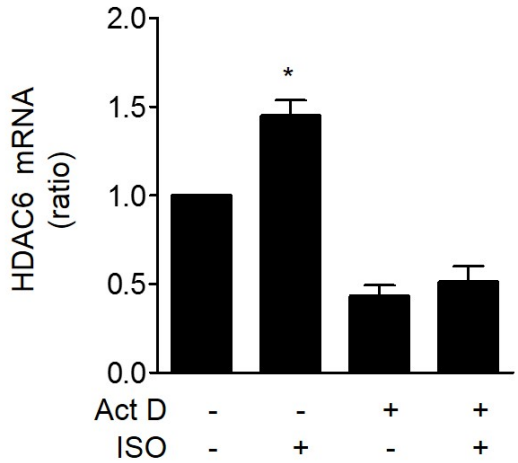


Figure 11. Effects of transcription inhibition on the isoproterenol-induced increase in HDAC6 RNA expression.

H1299 cells were pre-treated with 8 μM actinomycin D for 2 h before isoproterenol treatment (20 μM). The cells were harvested at 30 h for analysis of mRNA levels. The HDAC6 mRNA level was analyzed by quantitative real time PCR, normalized to GAPDH mRNA, and expressed as a ratio relative to the control level. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

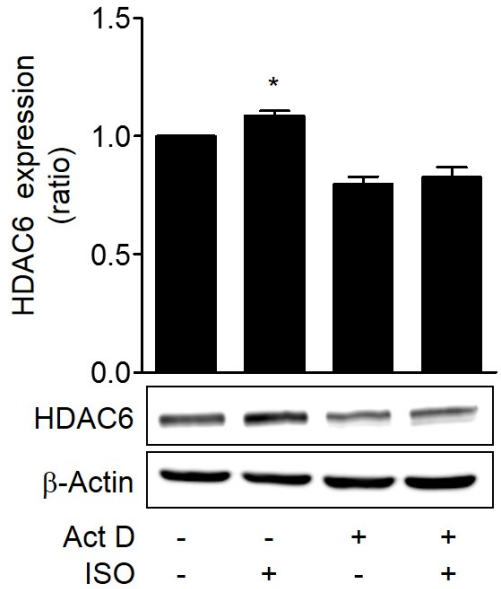


Figure 12. Effects of transcription inhibition on the isoproterenol-induced increase in HDAC6 protein expression.

H1299 cells were pre-treated with 8 μM actinomycin D for 2 h before isoproterenol treatment (20 μM). The cells were harvested at 48 h for western blot analysis. β-Actin was analyzed as a loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

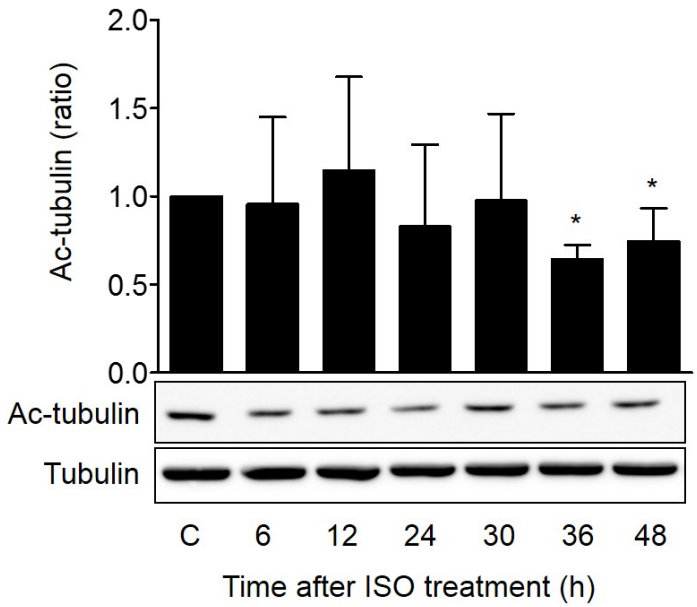


Figure 13. Effects of isoproterenol on the acetylation of α-tubulin.

H1299 cells were treated with 20 μM isoproterenol, and the levels of acetylated α-tubulin (Ac-tubulin) and total tubulin were assessed at the indicated time points by western blotting. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

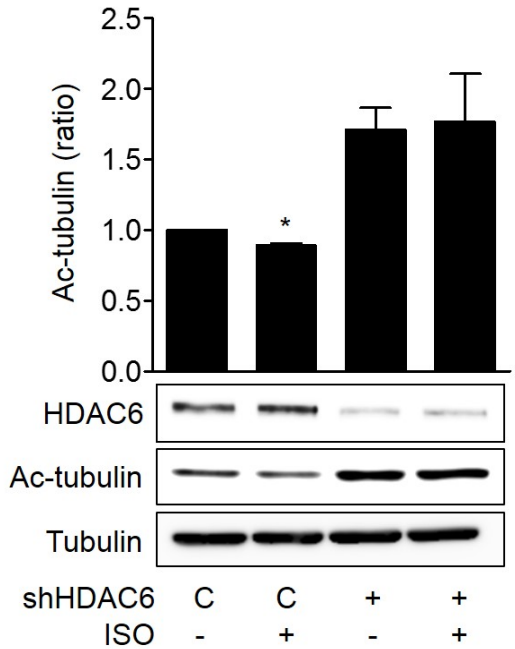
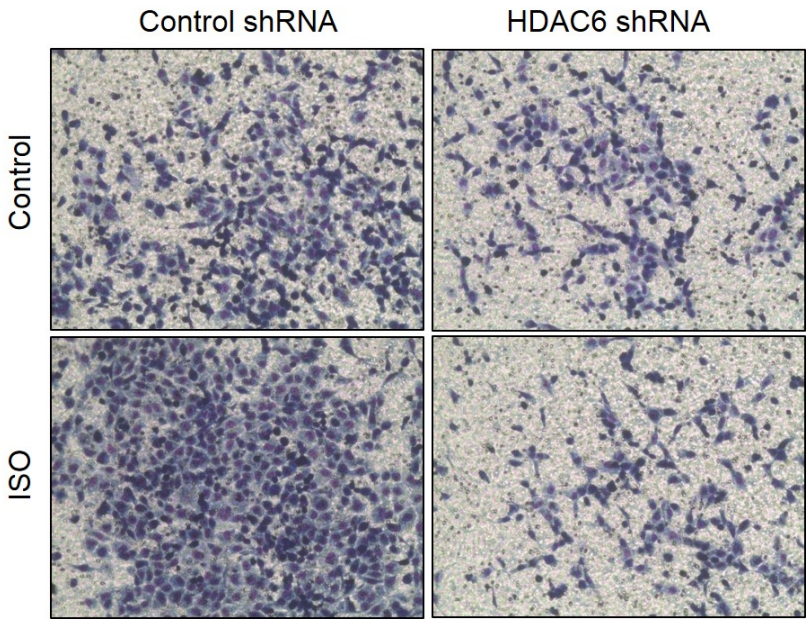


Figure 14. Effects of HDAC6 knock down on the isoproterenol-induced deacetylation of α-tubulin.

H1299 cells in 100 mm dishes were transfected with shRNA targeting HDAC6 or control shRNA (10 μg) and incubated for 24 h. Then, the cells were treated with 20 μM isoproterenol for an additional 48 h prior to harvesting for western blot analysis. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).


Control shRNA
HDAC6 shRNA
Control
ISO

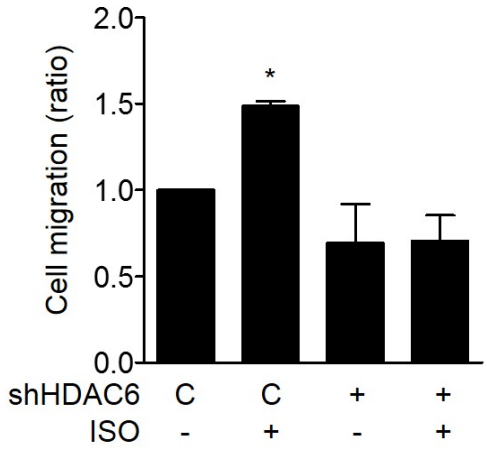

Cell migration (ratio)
2.0
1.5
1.0
0.5
0.0
*
shHDAC6 C C + +
ISO - + - +

Figure 15. Effects of isoproterenol on the migration of H1299 lung cancer cells.

A transwell migration assay was performed. In brief, H1299 cells were transfected with shRNA targeting HDAC6 or control shRNA, and the cells were seeded onto the upper chamber containing 20 μM isoproterenol. After 16 h, the migrated cells were fixed and stained, and representative photographs (100x magnification) of the migrated cells are presented. The migrated cells were counted in five different microscopic fields, and the averages were calculated. The values presented are the means ± the SDs of three independent experiments. Asterisks (*) indicate significant differences from the isoproterenol-untreated control ($P<0.05$, Mann-Whitney *U*-test).

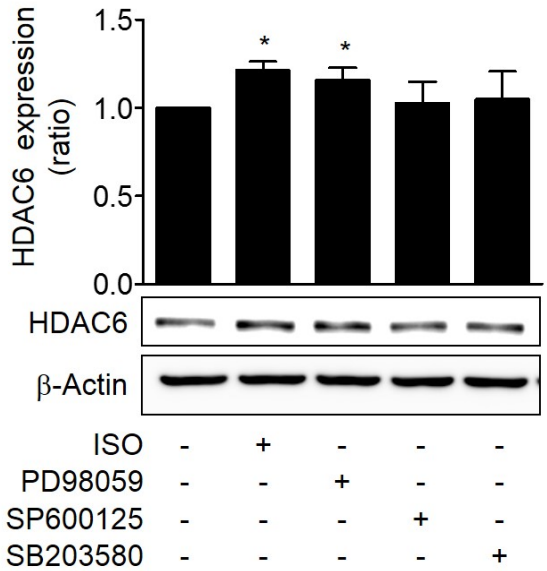


Figure 16. Effects of MAPK inhibitors on HDAC6 expression.

H1299 cells were treated with 20 μM isoproterenol (ISO), 20 μM PD98059, 10 μM SP600215, or 20 μM SB03580 for 48 h then HDAC6 expression was analyzed by western blotting and β-Actin was analyzed as a loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

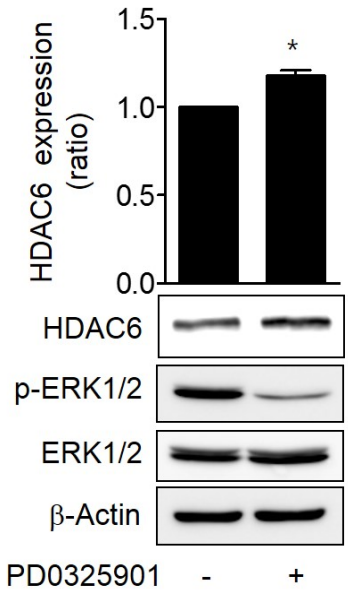


Figure 17. Effect of PD0325901 on HDAC6 expression in H1299 cells.

H1299 cells were treated with 1 μM PD0325901 for 48 h then HDAC6 expression was analyzed. β-Actin was analyzed as a loading control for western blot. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

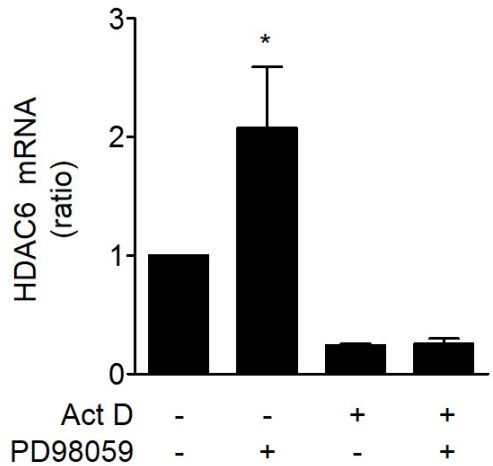


Figure 18. Effect of transcription inhibition on MEK inhibitor-induced increase in HDAC6 RNA.

H1299 cells were pre-treated with 40 nM actinomycin D for 2 h, then PD98059 was treated for 30 h mRNA was assessed. The HDAC6 mRNA level was analyzed by quantitative real time PCR, normalized to GAPDH mRNA, and expressed as a ratio relative to the control level. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

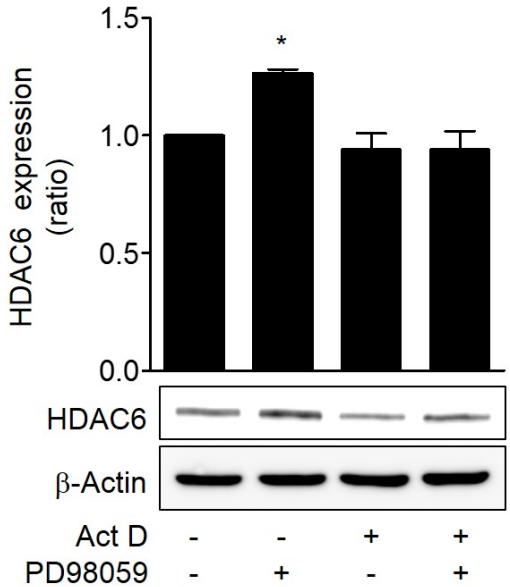


Figure 19. Effect of transcription inhibition on MEK inhibitor-induced increase in HDAC6 protein.

H1299 cells were pre-treated with 40 nM actinomycin D for 2 h, then PD98059 was treated for 46 h then HDAC6 expression was analyzed by western blot. β-Actin was analyzed as a loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

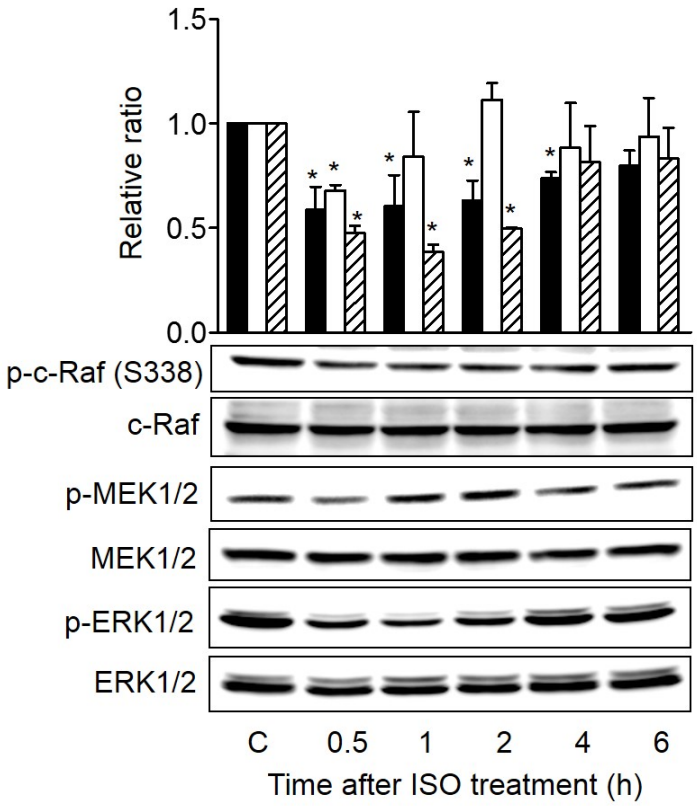


Figure 20. Effects of isoproterenol on c-Raf-MEK-ERK signaling.

H1299 cells were treated with isoproterenol for the indicated times, and the phosphorylations of c-Raf (filled bar), MEK (empty bar), and ERK (slant bar) were then analyzed by western blotting and densitometry. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

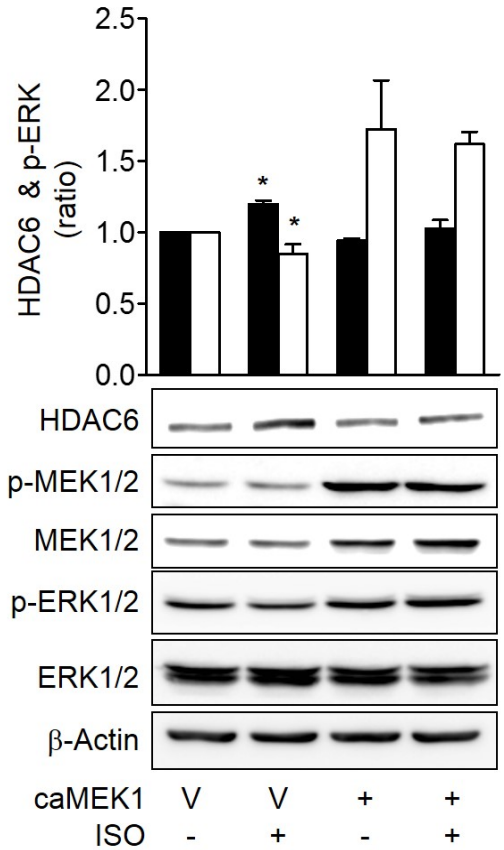


Figure 21. Effects of ERK activation on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with constitutively active MEK1 (caMEK1) for 24 h and were then treated with isoproterenol for 48 h prior to analysis. β-Actin was analyzed as a loading control. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the isoproterenol-untreated control ($P<0.05$, Mann-Whitney *U*-test).

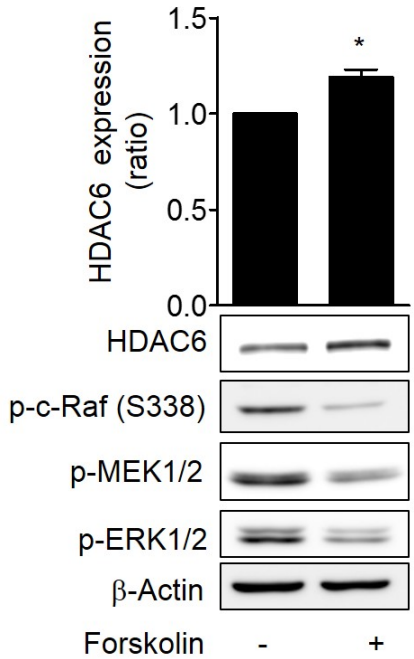


Figure 22. Effect of forskolin on HDAC6 expression and c-Raf-MEK-ERK pathway in A549 lung cancer cells.

A549 cells were treated with forskolin (20 μM) before HDAC6 expression was analyzed. β-actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

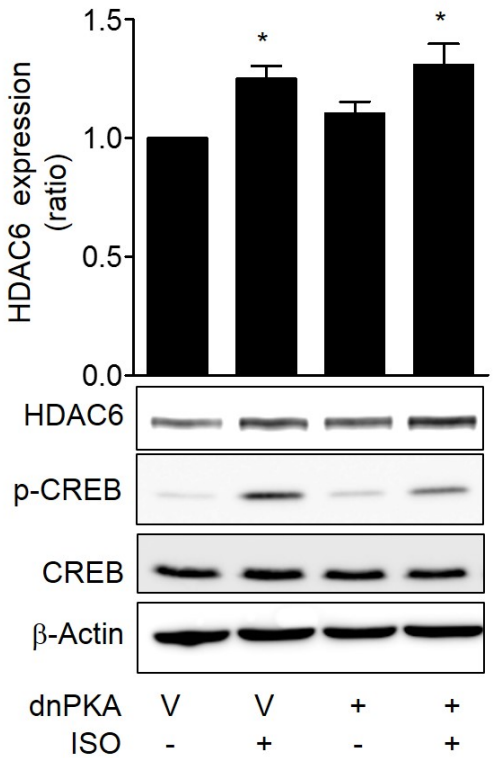


Figure 23. Effects of PKA inhibition on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with dominant negative PKA (dnPKA) for 24 h then, treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

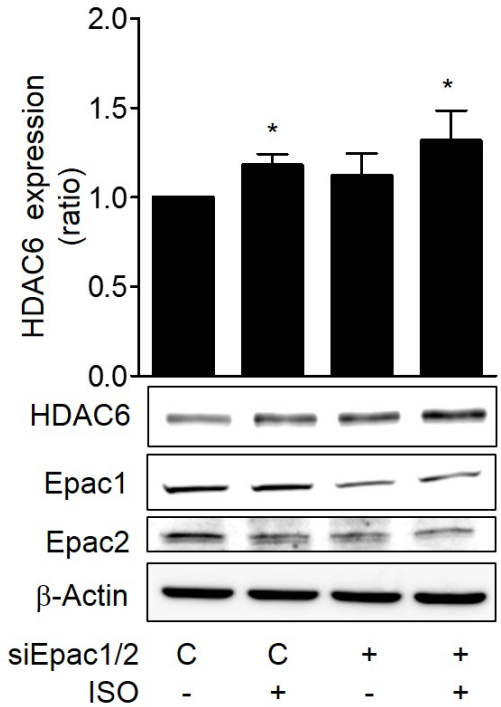


Figure 24. Effects of Epac knock down on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with Epac1 siRNA, Epac2 siRNA, scrambled control siRNA, incubated for 24 h, and then treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

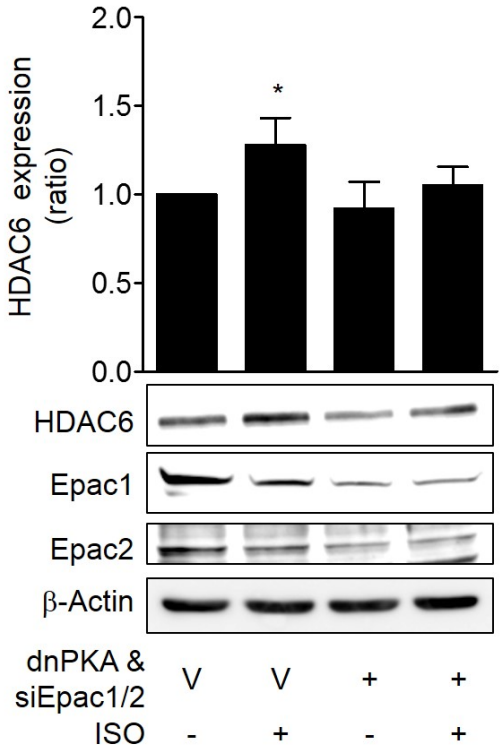


Figure 25. Effect of simultaneous PKA inhibition and Epac knock down on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with dominant negative PKA (dnPKA), Epac1siRNA, Epac2 siRNA, scrambled control siRNA, incubated for 24 h, and then treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

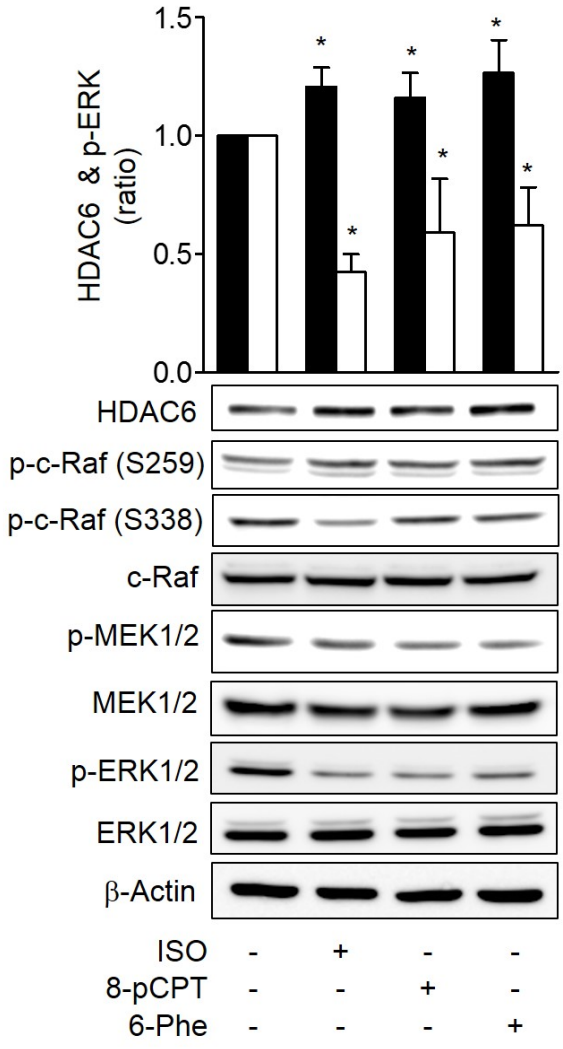

HDAC6 & p-ERK (ratio)
1.5
1.0
0.5
0.0
*
HDAC6
p-c-Raf (S259)
p-c-Raf (S338)
c-Raf
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
β-Actin
ISO - + - -
8-pCPT - - + -
6-Phe - - - +

Figure 26. Effects of Epac- and PKA-selective agonists on HDAC6 expression and c-Raf-MEK-ERK pathways.

H1299 cells were treated for 48 h with 20 μM ISO, 20 μM 8-pCPT-2' o-Me-cAMP (8-pCPT), EPAC activator, or 30 μM N6-Phenyl-cAMP (6-Phe) for 48 h before western blot analysis. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

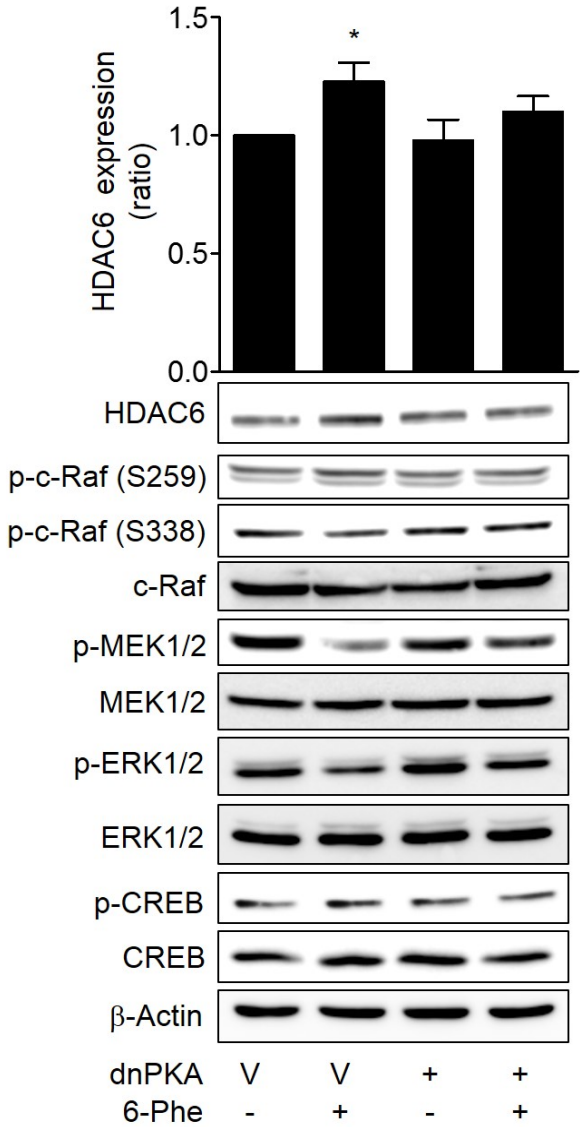

1.5
1.0
0.5
0.0
HDAC6 expression (ratio)
*
HDAC6
p-c-Raf (S259)
p-c-Raf (S338)
c-Raf
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
p-CREB
CREB
β-Actin
dnPKA V V + +
6-Phe - + - +

Figure 27. Effect of PKA inhibition on 6-Phe-induced HDAC6 expression and inhibition of c-Raf-MEK-ERK pathway.

H1299 cells were transfected with dominant negative PKA (dnPKA) for 24h then, treated with 30 μM N6-phenyl-cAMP (6-Phe) for 48 h. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

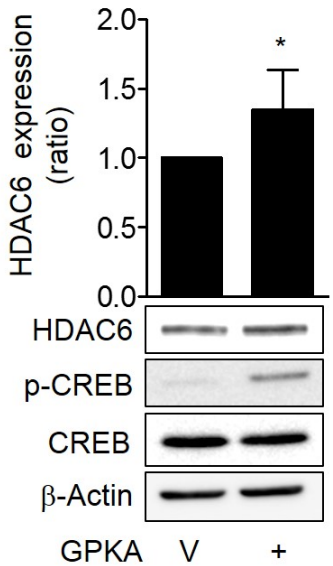


Figure 28. Effects of PKA activation on HDAC6 expression.

H1299 cells were transfected with the catalytic subunit of PKA (GPKA) or EGFP vector for 48 h. Then HDAC6 expression was analyzed by western blot. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

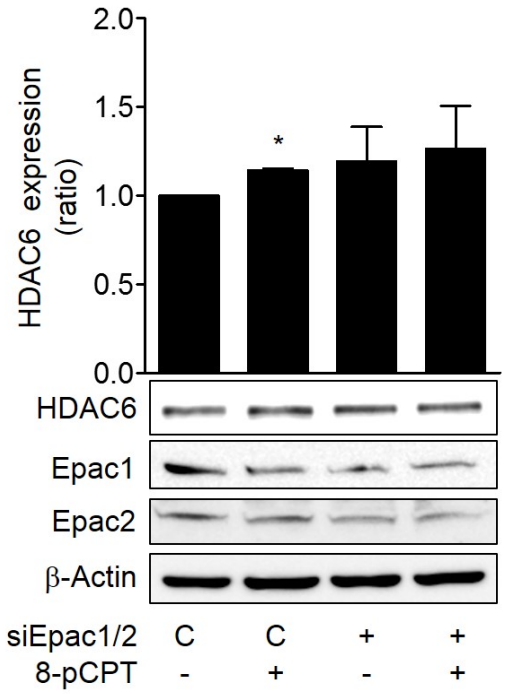


Figure 29. Effects of Epac knock down on 8-pCPT-cAMP-induced increase in HDAC6 expression.

Epac1 and 2 or control siRNA were transfected for 24 h. Then, 20 μM 8-pCPT-cAMP treatment was applied for 48 h prior to western blot analysis. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

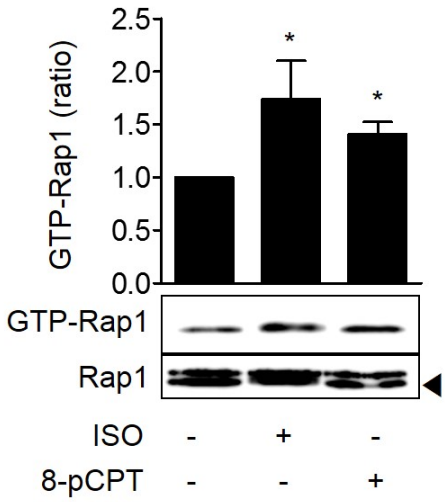


Figure 30. Effects of isoproterenol on Rap1 activation.

H1299 cells were treated with 20 μM isoproterenol or 20 μM 8-pCPT-cAMP for 30 min. The GTP-bound Rap1 was then pulled down using the immobilized Rap1-binding domain (RBD) of RalGDS, and western blot analysis was subsequently performed. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

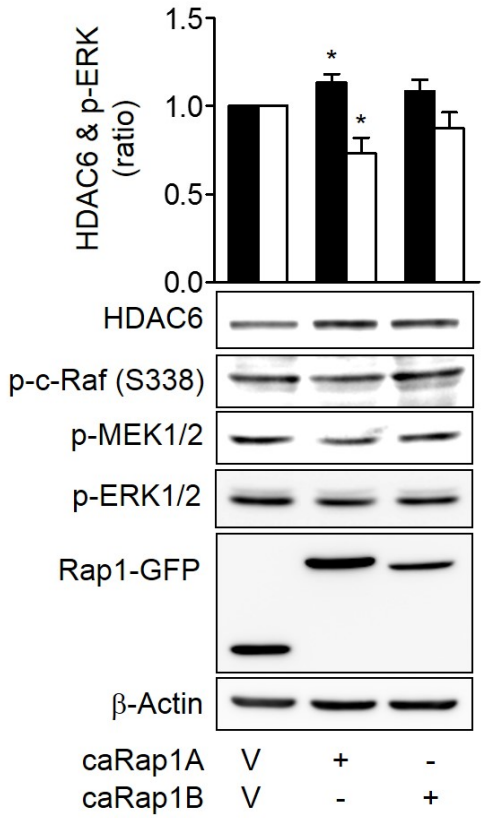

HDAC6 & p-ERK (ratio)
1.5
1.0
0.5
0.0
*
*
HDAC6
p-c-Raf (S338)
p-MEK1/2
p-ERK1/2
Rap1-GFP
β-Actin
caRap1A V + -
caRap1B V - +

Figure 31. Effects of Rap1 activation on ERK phosphorylation and HDAC6 expression.

H1299 cells were transfected with constitutively active Rap1A (caRap1A) and Rap1B (caRap1B), or EGFP control constructs (V) and incubated for 48 h prior western blot analysis. β-Actin was analyzed as a loading control for western blot. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

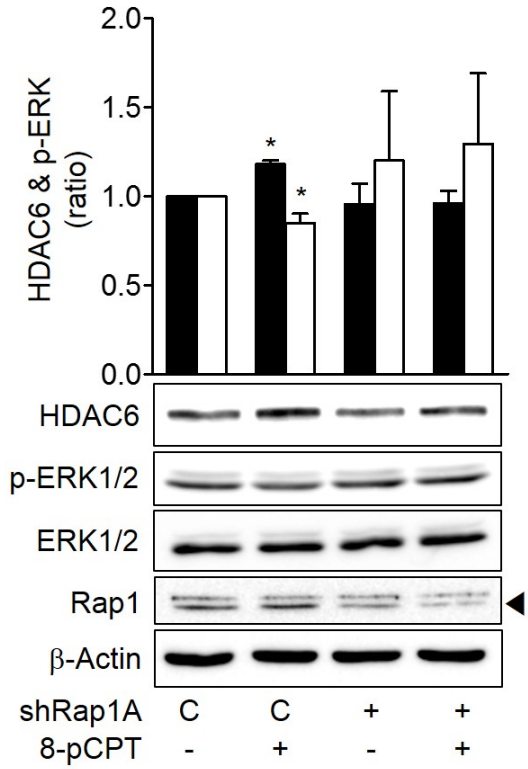


Figure 32. Effects of Rap1A knock down on 8-pCPT-cAMP-induced ERK phosphorylation and HDAC6 expression.

H1299 cells were transfected with Rap1A shRNA, incubated for 24 h, and then treated with 20 μM 8-pCPT-cAMP for 48 h. β-Actin was analyzed as a loading control for western blot. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

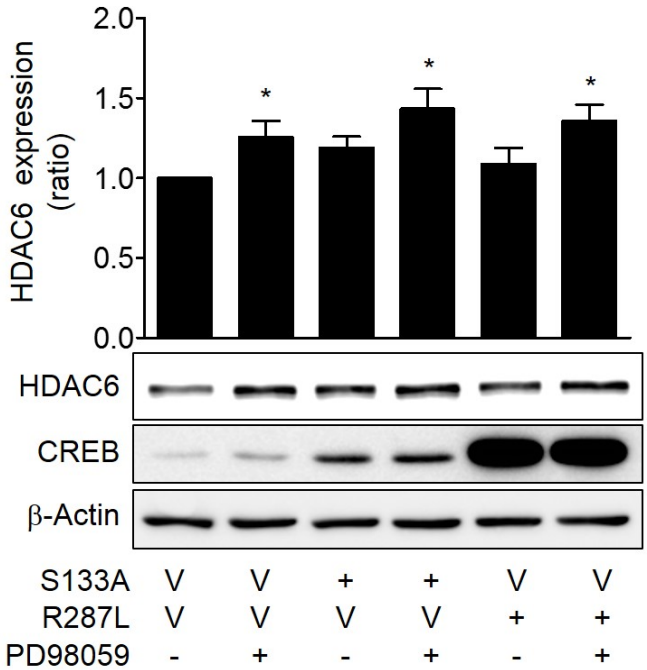


Figure 33. Effects of dominant negative CREB on PD98059-induced HDAC6 expression.

H1299 cells were transfected with dominant-negative CREBs (S133A, R287L), respective control vectors (V) for 24 h then 20 μM PD 98059 was treated for 48 h. Then HDAC6 expression was analyzed by western blot. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

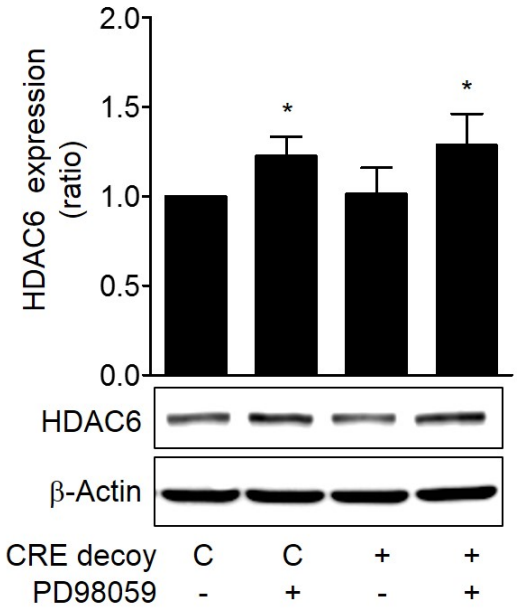


Figure 34. Effect of inhibition of CREB binding to CRE on PD98059 induced HDAC6 expression.

H1299 cells were transfected with oligonucleotides (CRE decoy or CRE control (C)) for 24 h then PD 98059 was treated for 48 h western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

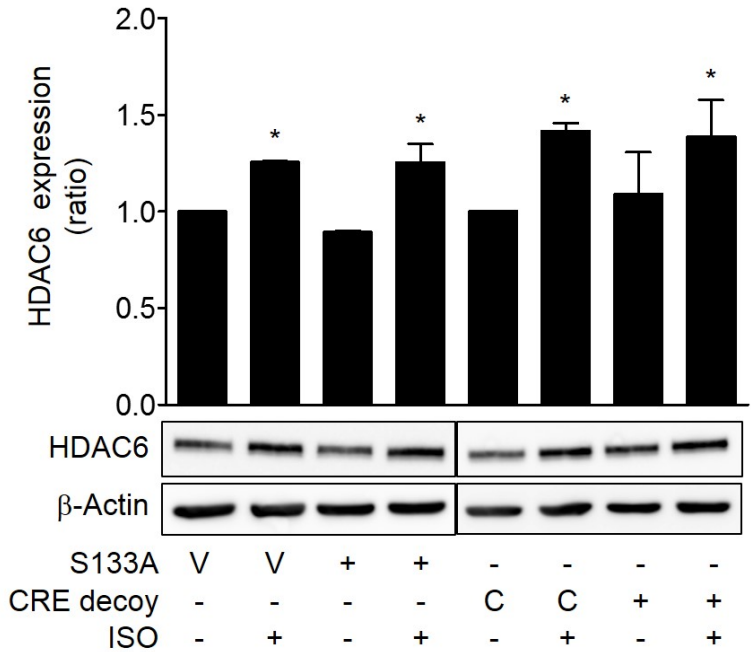


Figure 35. Effects of CRE decoy oligonucleotides CREB on isoproterenol-induced HDAC6 expression.

H1299 cells were transfected with dominant-negative CREBs (S133A, R287L), respective control vectors (V), or CRE decoy and control (C) oligonucleotides and then incubated for 24 h. The cells were then treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

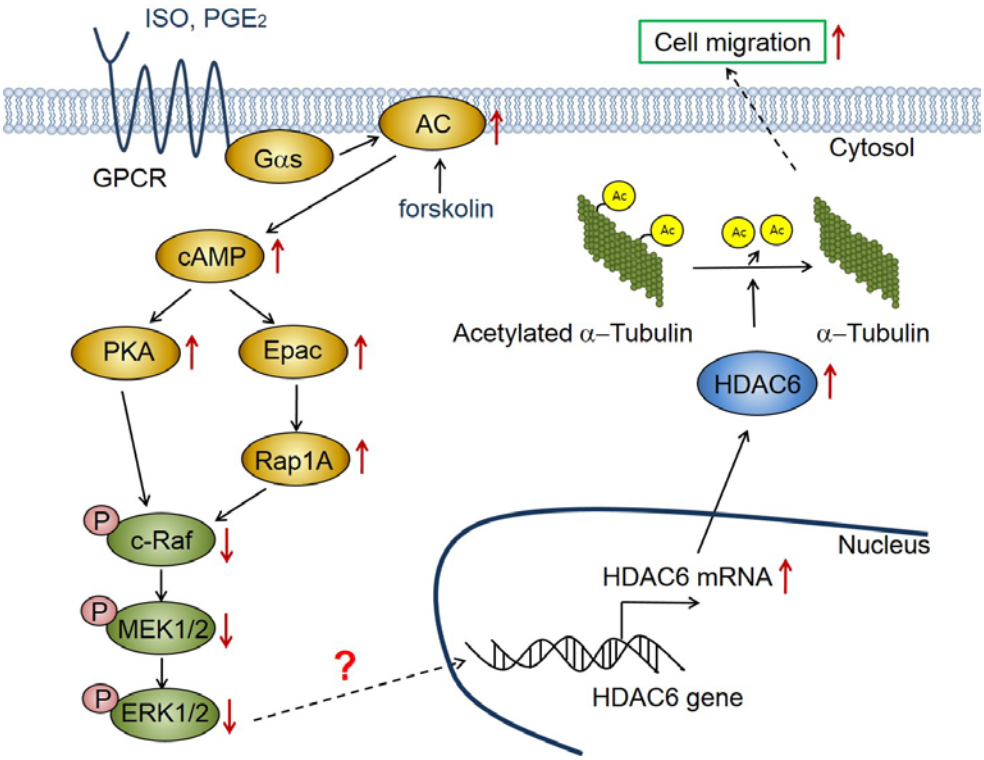


Figure 36. A suggested mechanism by which isoproterenol increases HDAC6 expression in human lung cancer cells.

Isoproterenol increases HDAC6 expression via the PKA- and Epac-mediated inhibitions of c-Raf-MEK-ERK, which result in increased cell migration. The solid lines indicate proven signaling pathways, and the dotted lines indicate potential signaling pathways. GPCR, G-protein-coupled receptor.

Discussion

This study investigated the effects of stress signals on HDAC expression and their underlying mechanisms in human lung cancer cells. We found that isoproterenol signaling increases HDAC6 expression by inducing transcription through a PKA/Epac/ERK-dependent pathway and that the isoproterenol-induced increase in HDAC6 expression stimulates the migration of H1299 lung cancer cells (Figure 36).

Our finding that isoproterenol signaling increased the expression of HDAC6, which stimulated the migration of lung cancer cells, is supported by the result that treatment with isoproterenol increased the expressions of HDAC6 mRNA and protein without affecting the expressions of other class II HDACs in H1299 and A549 human lung cancer cells and that isoproterenol treatment caused a decrease in the acetylation of α-tubulin and an increase in cell migration in an HDAC6-dependent manner. Isoproterenol is an isopropyl analog of epinephrine, which is a major stress hormone, and activates β-adrenergic receptors, which are the receptors for

epinephrine and norepinephrine. Therefore, our findings suggest that stress might increase HDAC6 expression, which might in turn stimulate the migration of cancer cells. HDAC6 has been reported to deacetylate Hsp90 to regulate glucocorticoid receptor chaperone dynamics in the brain, which provides a promising strategy to reduce the harmful socioaffective effects of stress and glucocorticoids (36, 37). Chronic social stress has been found to decrease the expression of histone deacetylase 2 in the mouse brain (35). However, to the best of our knowledge, this is the first report to reveal that the isoproterenol stress signal increases HDAC6 expression in lung cancer cells.

HDAC6 is predominantly localized in the cytoplasm and regulates many important biological processes, including cell migration, immune synapse formation, transcription, cell proliferation and death, and the degradation of misfolded proteins (45). HDAC6 deacetylates α-tubulin and thus increases microtubule dynamics to increase cell motility (46), and it also modulates actin-dependent cell movement by altering the acetylation status of cortactin (47). Thus, we suggest that the increase in HDAC6 expression by isoproterenol stimulate the migration of lung cancer cells via the deacetylations of

α-tubulin and cortactin. A similar role of HDAC6 on cell migration and invasion has been reported in hepatocellular carcinomas (48). Stress signals, such as norepinephrine, have been reported to enhance invasive potential via the up-regulation of matrix metalloproteinases in ovarian and prostate cancer cells (3, 4). Our paper presents a novel mechanism, i.e., the up-regulation of HDAC6 expression, by which stress signals might stimulate the invasiveness of cancer cells. In addition, HDAC6 has been reported to deacetylate Hsp90 to regulate glucocorticoid receptor chaperone dynamics in the brain, which provides a promising strategy to reduce the harmful socioaffective effects of stress and glucocorticoid (36, 37). Furthermore, because HDAC6 are known to interact a variety of proteins (49), isoproterenol-induced HDAC6 expressions could regulate the other biological responses involved in cancer progression. HDAC6 can shuttle into the nucleus and deacetylate histone in vitro and in vivo, and therefore the effect of isoproterenol-induced HDAC6 expression on epigenetic regulation need to be further investigated.

We found that isoproterenol increases HDAC6 by activating a cAMP signaling pathway that involves both PKA and Epac, both of

which inhibit the c-Raf-MEK-ERK pathway in lung cancer cells. This finding is based on the result that HDAC6 expression was increased when cAMP signaling was activated by the expression of constitutively active Gαs or treatment with Gαs-coupled receptor agonists (i.e., PGE_2 and isoproterenol) and an adenylyl cyclase activator (forskolin) in the H1299 and A549 lung cancer cells. Similar to epinephrine and norepinephrine, isoproterenol binds β-adrenergic receptors to trigger the sequential activation of stimulatory G proteins, adenylyl cyclases, PKA, and Epac signaling (50). This finding suggests that HDAC6 expression might be increased not only by the stress signal isoproterenol but also by other signals that increase cAMP concentrations, such as phthalates (51). Additionally, cAMP signaling has been reported to stimulate HDAC4 activity in macrophages (52) and to decrease sirtuin 6 expression in lung cancer cells (53). Thus, we suggest that stress signals might regulate histone acetylation and gene expression by various ways via activating cAMP signaling.

cAMP signaling regulates various cellular responses by activating three major cAMP effector molecules: cAMP-dependent protein kinase (PKA), exchange factor directly activated by cAMP

(Epac), and cyclic-nucleotide-gated ion channels (CNG) (54). This study revealed that both PKA and Epac mediate the HDAC6-increasing effect of isoproterenol by demonstrating that the inhibition of either PKA or Epac alone did not abolish the effect of isoproterenol on HDAC expression, but the simultaneous inhibition of both PKA and Epac abolished this effect. Furthermore, treatment with either a PKA- or Epac-selective agonist increased HDAC6 expression. The involvement of PKA was further evidenced by the demonstration that the expression of the PKA catalytic subunit increased HDAC6 expression, and Epac-selective agonist elicited increased HDAC6 expression in via a Rap1A-dependent pathway.

In the study on the mechanisms by which PKA and Epac increased HDA6 expression, the PKA and Epac pathways were found to mediate the HADC6-increasing effect of isoproterenol by inhibiting c-Raf-MEK-ERK signaling. This finding is supported by the results that treatment with isoproterenol and selective agonists of PKA or Epac together inhibited the c-Raf-MEK-ERK signaling pathway, the inhibition of the c-Raf-MEK-ERK signaling pathway increased the HDAC6 expression, and isoproterenol increased HDAC6 expression in an ERK inhibition-dependent manner. ERK is

a member of the mitogen-activated protein kinase (MAPK) family and participates in the regulation of various processes, including cell migration and proliferation and transcription. ERK is activated by following the classical cascade of consecutive activating phosphorylation events: Raf phosphorylates and activates MEK, and activated MEK phosphorylates and activates ERK (55). The cAMP and MAPK pathways do not act independently; rather, multiple forms of cross-talk between these pathways can occur (56). As shown in our paper, cAMP signaling has previously been reported to inhibit the c-Raf-MEK-ERK pathway by decreasing activating phosphorylation at Ser-338 and increasing inactivating phosphorylation at Ser-259 on the c-Raf protein (27, 57, 58). The Rap1 protein also inhibits c-Raf activation via the sequestration of c-Raf from Ras through competition for Ras (59, 60).

In the present study, we found that isoproterenol increases HDAC6 expression by inhibiting the c-Raf-MEK-ERK pathway, but the mechanism by which the inhibition of ERK induces HDAC6 expression in a CREB-independent pathways requires elucidation. Phthalates have been reported to increase HDAC6 expression via PKA-dependent CREB phosphorylation, which results in increased

CREB binding to a CRE site of the HDAC6 promoter region (51). However, the increase in HDAC6 expression induced by isoproterenol or PD98059 was not abolished by blocking the binding of active CREB to CRE sites using dominant negative CREBs and CRE decoy oligonucleotides in our study, which suggests other mechanisms in isoproterenol-induced increase in HDAC6 expression in lung cancer cells. The ETS transcription factor Erg has been reported to regulate the expression of HDAC6 in human vascular endothelial cells (61), and Erg has domains that act as docking platforms for mitogen-activated protein (MAP) kinases; such docking leads to phosphorylation and the enhancement of transactivation activity (62). Thus, we speculated that ERK signaling might inhibit Erg activation or induce the expression of genes that repress HDAC6 gene transcription.

Conclusion

The following conclusions were obtained from this study.

1. Isoproterenol increases the migration of H1299 non-small cell lung cancer cells by HDAC6-induced decrease in acetylation of α-tubulin.

2. Isoproterenol stress signaling increases HDAC6 expression via a PKA/Epac/ERK-dependent pathway.

This study suggests that stress signals may stimulate the migration of cancer cells by increasing the expression of HDAC6.

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국문 초록

임정아
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스트레스는 암의 성장, 진행, 전이와 관련 있다. 에피네프린 신호는 스트레스 상태에서 활성화된다. 에피네프린의 아날로그인 이소프로테레롤은 베타 아드레날린 수용체와 결합하여 활성화시켜 cAMP 산물을 촉진시키고, 다양한 세포의 대사와 유전자 발현을 조절한다. 알파 튜불린은 히스톤 탈아세틸화효소 6 (HDAC6)에 의해 탈아세틸화되며, 미세소관의 역학에 영향을 주어 세포의 운동성을 조절한다. 이 연구에서는 스트레스 신호가 비소세포성 폐암 세포주에서 HDAC6의 발현 조절을 통해 세포의 이동에 영향을 주는 기전을 확인하고자 한다. H1299 폐암 세포주에 이소프로테레놀을 처리하였을 때 알파 튜불린의 아세틸화를 감소하였으며, 세포의 운동성을 촉진되었다. 또한 이소프로테레놀의 처리는 HDAC6의 발현이 증가시켰다. 이소프로테레놀에 의해 활성화된 세포의 이동은 HDAC6 를 감소시켰을 때 억제되었다. Protein kinase A (PKA) 활성제인 N6-phenyl-cAMP 나 exchange protein activated by cAMP (Epac)의 활성제인 8-pCPT-2'-O-Me-cAMP (8-pCPT) 를 처리하였을 때 HDAC6 의 발현이 증가하였다. 이소프로테레놀이나 8-pCPT을 처리하였을 때 EPAC의 하위인 Rap1의 활성이 증가하였다. 항시 활성형

Rap1A에 의해 HDAC6의 발현이 증가하였고, Rap1A의 발현을 저해하면, 이소프로테레놀의 의해 증가된 HDAC6의 발현이 감소하는 것을 확인하였다. H1299 세포에서 이소프로테레놀은 External signal-activated kinase (ERK) 를 억제하였다. ERK의 억제는 HDAC6의 발현을 증가시켰고, 항시 활성형 MAPK kinase (MEK1)에 의해서는 이소프로테레놀에 의해 증가된 HDAC6의 발현이 감소하였다. 따라서 이소프로테레놀은 PKA/Epac/ERK 신호 전달계를 통해 HDAC6의 발현을 증가시키고, 이소프로테레놀에 의해 유도된 HDAC6의 발현이 폐암세포의 이동성을 증가시킨다는 결론을 내렸다. 이 연구는 스트레스 신호가 HDAC6의 발현을 증가시켜 암세포의 이동성을 촉진할 수 있다는 것을 보여준다.

주요어: 스트레스 신호, Histone deacetylase 6 (HDAC6), Extracellular -regulated kinase 1/2, 세포 이동성

학번 : 2011-30621

이학박사 학위논문

Molecular mechanism for cAMP signaling to regulate HDAC6 expression in non-small cell lung cancer

cAMP 신호전달계가 폐암세포주에서 HDAC6의 발현을 조절하는 분자기전

2016년 2월

서울대학교 대학원

협동과정 종양생물학 전공

임 정 아

Abstract

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Stress conditions are correlated with tumor growth, progression and metastasis. Epinephrine signaling is activated during stress conditions. Isoproterenol, an epinephrine analog, binds and activates β-adrenergic receptor to stimulate cAMP production and regulate various cellular metabolism and gene expression. α-Tubulin is deacetylated by histone deacetylase 6 (HDAC6), which affects the microtubule dynamics to regulate cell migration. This study investigated the mechanism for stress signals to modulate the migration of non-small cell lung cancer cells via regulation of HDAC6 expression. Treatment of H1299 lung cancer cells with isoproterenol decreased the acetylation level of α-tubulin and stimulated cell migration. Isoproterenol treatment also increased the expression of HDAC6. Isoproterenol-stimulated cell migration was blocked by

knockdown of HDAC6. Treatment with N6-phenyl-cAMP, a selective activator of protein kinase A (PKA), and 8-pCPT-2'-O-Me-cAMP (8-pCPT), a selective activator of exchange protein activated by cAMP (Epac), increased HDAC6 expression. Isoproterenol and 8-pCPT increased Rap1 activity, which acts downstream of Epac. Constitutively active Rap1A increased HDAC6 expression and the knockdown of Rap1A decreased isoproterenol-induced HDAC6 expression. Isoproterenol inhibited External signal-activated kinase (ERK) in H1299 cells. Inhibition of ERK increased HDAC6 levels, and the expression of constitutively active MAPK kinase (MEK1) decreased isoproterenol-induced HDAC6 expression. It is concluded that isoproterenol increases HDAC6 expression via PKA/Epac/ERK-dependent pathway, and isoproterenol-induced HDAC6 expression increases the migration of lung cancer cells. This study suggests that stress signal can stimulate the migration of cancer cells by increasing the expression of HDAC6.

Keywords: cAMP; cell migration; histone deacetylase 6; isoproterenol; lung cancer; stress

Student Number : 2011-30621

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Introduction

1. The stress response and its associated signaling pathways

Stress affects the immune system, increases the production of pro-inflammatory cytokines, and also promotes depression, anxiety and drug-seeking behavior (1). It can influence the response to infectious disease, inflammation, and aging (2). Stress causes a fight-or-flight response, inducing the production of catecholamine neurotransmitters such as epinephrine and norepinephrine by the sympathetic nervous system and adrenal medulla (3, 4). Epinephrine binding to β-adrenergic receptor activates Gs protein (5), which activates adenylyl cyclase and increases the intracellular concentration of 3′, 5′-cyclic adenosine monophosphate (cAMP). An elevated concentration of cAMP regulates cellular functions including metabolism, differentiation, gene expression, and migration. cAMP regulates the protein kinase

A (PKA) and exchange protein directly activated by cAMP (Epac) pathways (6). PKA phosphorylates cAMP response element binding protein (CREB) (7, 8) and phosphorylated CREB promotes many kinds of target gene activation. Epac binds and activates a downstream effector, the Ras-like small GTPase Rap, which regulates cell adhesion, proliferation, and apoptosis (9).

2. G protein

2.1 Heterotrimeric G protein

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are composed of 3 subunits (α, β and γ) and exist in almost all cells. They induce signaling responses and regulate various biological phenomena such as cell growth, differentiation, and metabolism. When unstimulated, the G protein is bound to guanine diphosphate (GDP), but upon activation by an extracellular ligand, GDP is exchanged for guanine triphosphate (GTP), resulting in the G protein undergoing a conformational change to its active form (10).

When GTP binds to the Gα subunit, the Gβγ s subunit dissociates from Gα (11). The α subunit of G proteins each consist of 390-395 amino acids, and they are divided into 4 classes: Gs, Gi, Gq, and G12 (12). Gs stimulates adenylyl cyclase and regulates Ca^{2+} channels. Gi inhibits adenylyl cyclase, regulates K^{+} and Ca^{2+} channels, and activates cyclic guanine monophosphate (cGMP) phosphodiesterase. Gq activates phospholipase C (PLC), and G12 regulates Na^{+}/K^{+} exchange (13). The β subunit has five different subunits and γ has 12 different subunits. Gβγ dimers activate effectors including PLCβ2, β3, β-adrenergic receptor kinase and phosphoinositide 3-kinase (PI3 kinase) (14).

2.2 3', 5'-cyclic adenosine monophosphate (cAMP)

3′, 5′-cyclic adenosine monophosphate (cAMP) was first identified as a ubiquitous second messenger regulated by adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE) (15, 16). When a ligand binds to a G protein coupled receptor (GPCR), it activates AC to

produce cAMP from ATP. Intracellular cAMP regulates various cellular functions including metabolism, differentiation, gene expression, cell growth and division, apoptosis, and migration. cAMP regulates several effectors, including PKA, Epac, and the cyclic nucleotide-gated ion channels (Figure 1) (6, 17, 18). cAMP also undergoes cross-talk with many intracellular signaling molecules.

2.3 cAMP-dependent protein kinase A (PKA)

Inactive PKA consists of two catalytic subunits and two regulatory subunits. Two classes of regulatory subunits (RI and RII) and three isoforms of catalytic subunits (Cα, Cβ and Cγ) have been identified. When cAMP binds to the regulatory subunits of PKA to activate PKA signaling, the regulatory subunits undergo conformational changes and are released from the catalytic subunits, which then phosphorylate their substrates: cAMP response element binding protein (CREB), cAMP response element binding modulator (CREM), and activating transcription factor 1 (ATF1). The phosphorylation

of these substrates is essential for them to interact with the transcriptional coactivators, CREB-binding protein (CBP) and p300 (15, 19).

2.4 Exchange protein directly activated by cAMP (Epac)

Epac binds and activates downstream effectors including the Ras-like small GTPases Rap1 and Rap2 to regulate cell adhesion, proliferation, and apoptosis (9). Epac has two isoforms, Epac1 and Epac2, which share a high degree of sequence homology and both have a regulatory region at the N-terminus and a catalytic region at the C-terminus (6). The expression of Epacs has been verified in many cell types. Epac1 is highly expressed in the thyroid, kidney, ovary, and skeletal muscle. Epac2 is mostly expressed in the brain and adrenal gland (20). Downstream of Epacs, Rap1 is regulated by many cellular processes and activated by intracellular molecules including cAMP (21). PKA also activates Rap1, but in a cAMP-independent manner (6, 22).

3. Epigenetics and histone modification

Gene expression is usually regulated by how tightly DNA is packaged in eukaryotic cells. The nucleosome, a chromatin subunit, is composed of an octamer of 4 core histones, comprising 2 H2A/H2B dimers and an H3/H4 tetramer. DNA is surrounded by this nucleosome. In resting cells, DNA is compacted to prevent binding of transcription factors; however, this compact structure can be partly unpacked via posttranslational modification of the histone proteins, allowing transcription factors to bind to the DNA and initiate gene transcription (23).

Histone modifications include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, adenosine diphosphate–ribosylation, deimination, and proline isomerization. Many kinds of enzymes are involved in regulating each type of modification. Among these modifications, acetylation is the best understood (24).

3.1 Histone acetyltransferase (HAT)

Histone acetylation is a reversible process and it regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs are divided into three families; Gcn5-related N-acetyltransferase, MYST, and CBP/p300. Acetylation at the H3 K56 residue was recently reported. The K56 residue is located in the major groove of the DNA so it may be involved in mediating direct interactions between DNA and H3 (24, 25).

3.2 Histone deacetylase (HDAC)

Histone deacetylases (HDACs) are divided into two families; classical HDACs and NAD^+-dependent HDACs SIR2 family. . Classical HDACs are divided into three subclasses based on their homology with yeast proteins. The class I HDACs (HDAC1, 2, 3, and 8) are usually localized in the nucleus. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) are predominantly localized in the cytoplasm and can translocate to the nucleus (23) (Figure 2). HDAC11 is in class IV, which

has recently been identified (26).

3.3 Histone deacetylase 6 (HDAC6)

Histone deacetylase 6 (HDAC6) belongs to HDAC class IIb and is the best characterized of the HDACs that have 2 tandem deacetylase domains. HDAC6 is predominantly localized in the cytoplasm because it has a nuclear export domain at the N-terminus and a SE14 domain at the C-terminus (27). Also, there is a zinc finger ubiquitin binding domain at the C-terminus of HDAC6, which binds with ubiquitin to regulate misfolded proteins (Figure 3) (28). Unlike other HDACs, HDAC6 deacetylates non-histone proteins, such as α-tubulin, Hsp90, cortactin, and Ku70. Therefore, it regulates many important biological processes including cell migration, inflammation, proliferation, and apoptosis (Figure 4). α-tubulin was the first identified substrate of HDAC6 and the acetylation of α-tubulin regulates microtubule dynamics to promote cell migration (29).

4. Mitogen-activated protein kinases (MAPKs)

MAPKs are serine- and threonine-specific kinases that regulate many cellular functions including gene expression, cell cycle control, and apoptosis. MAPKs are activated by a classical cascade of consecutive phosphorylation events. MAPK kinase kinases (MAPKKKs) phosphorylate MAPK kinases (MAPKKs), which phosphorylate and activate MAPKs. There are three MAPK subfamilies: the extracellular signal-related kinases (ERK1 and 2); the c-Jun N-terminal kinases (JNK1, 2, and 3); and the p38 enzymes (p38α, p38β, p38γ and p38δ)(Figure 5) (30).

4.1 Extracellular signal-related kinases (ERKs)

ERKs are widely expressed and regulate many cellular functions including gene transcription, proliferation, and differentiation. Various stimuli can activate the ERK pathway, such as cytokines, growth factors, and GPCR activators. The small GTP-binding protein Ras is an upstream regulator of ERK activation. Ras phosphorylates and activates a MAPKKK

(Raf), which phosphorylates a MAPKK (MEK), which phosphorylates and activates ERK. The ERK signaling pathway regulates proliferation through downstream targets including NF-kB, Ets-1, AP-1 and c-Myc. The ERK signaling pathway is abnormally activated in malignantly transformed cells and ERK inhibitors are used in cancer therapy. ERK signaling interacts with the phosphoinositide 3-kinase/Akt pathway to regulate the balance between cellular proliferation and apoptosis (30, 31). The ERK pathway and its interactors include important therapeutic targets.

5. Tumor cell migration, invasion and metastasis

Metastasis is an important process for cancers and causes 90% of the deaths ascribed to solid tumors (32). Tumor metastasis occurs through a complicated and multistep process. Understanding the mechanisms and methods for the inhibition of metastasis are essential for improving cancer therapy. Cancer cell migration is an initial step of invasion and tumor metastasis. The general mechanism of migration is

divided into 4 steps: protrusion, adhesion, contraction, and retraction. Cell migration occurs via a microtubule-dependent and actin-dependent pathway. Microtubules regulate cell adhesion to the extracellular matrix and the disassembly of focal adhesions (33). The stabilization of microtubules could be associated with the posttranslational modification of tubulin. Acetylated tubulin is observed in stable microtubules and its acetylation is completely reduced in the fibroblast leading edge, which is involved in cell movement (29). In the actin-dependent pathway, there are key regulatory proteins including Wiskott-Aldrich syndrome protein family proteins, Arp2/3 complex, LIM-kinase, cofilin and cortactin (34).

6. Stress and HDACs

Stress signaling involves HDACs. HDAC1 was reported to be involved in the regulation of kappa-opioid receptor (KOR) expression by behavioral stress (1), chronic social stress was associated with decreased levels of HDAC2 in the nucleus accumbens in mice (35), and HDAC6 inhibition

was found to induce proresilience and antidepressant interventions (36). In addition, HDAC6 regulates glucocorticoid receptor chaperone, which is activated under conditions of psychosocial stress (37). Therefore, stress signaling might affect tumor growth and progression by the epigenetic control of gene expression.

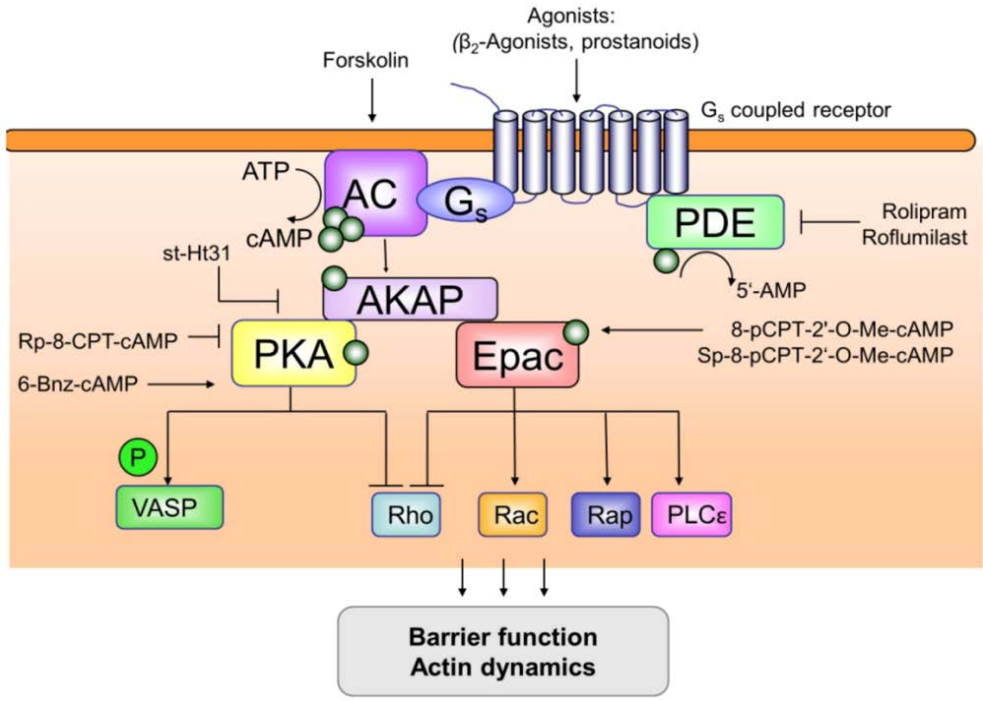


Figure 1. cAMP signaling pathway (17)

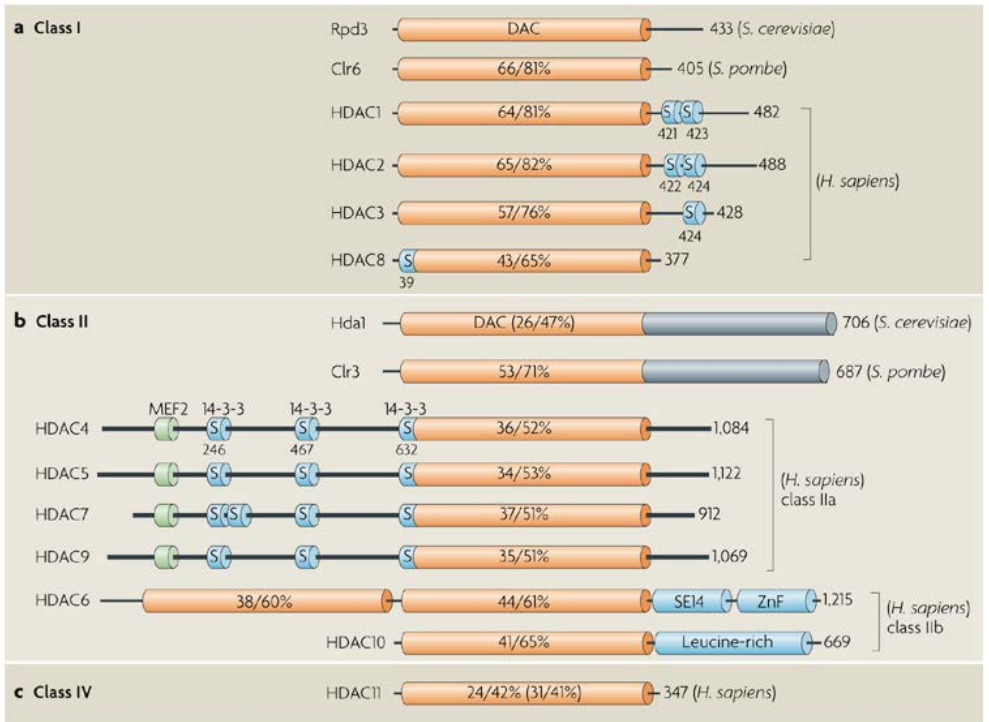


Figure 2. Domain of classical HDAC families (38)

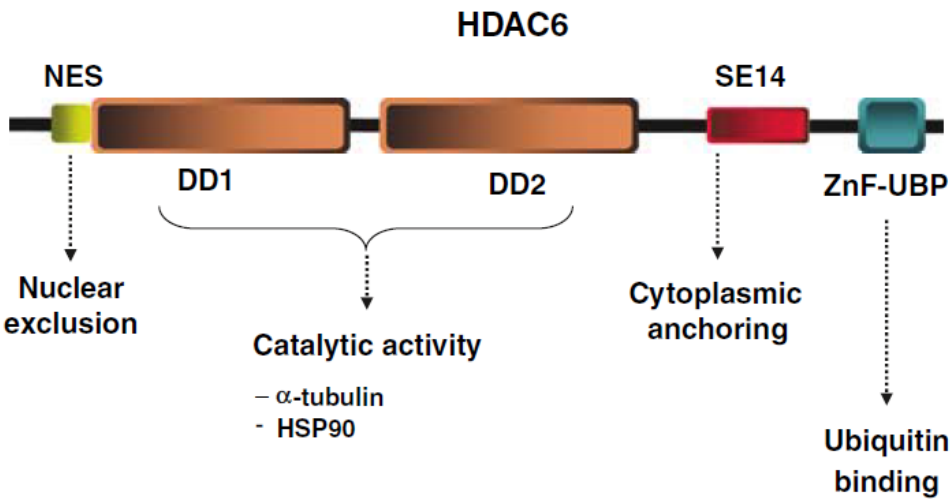


Figure 3. Functional domains of HDAC6 (39)

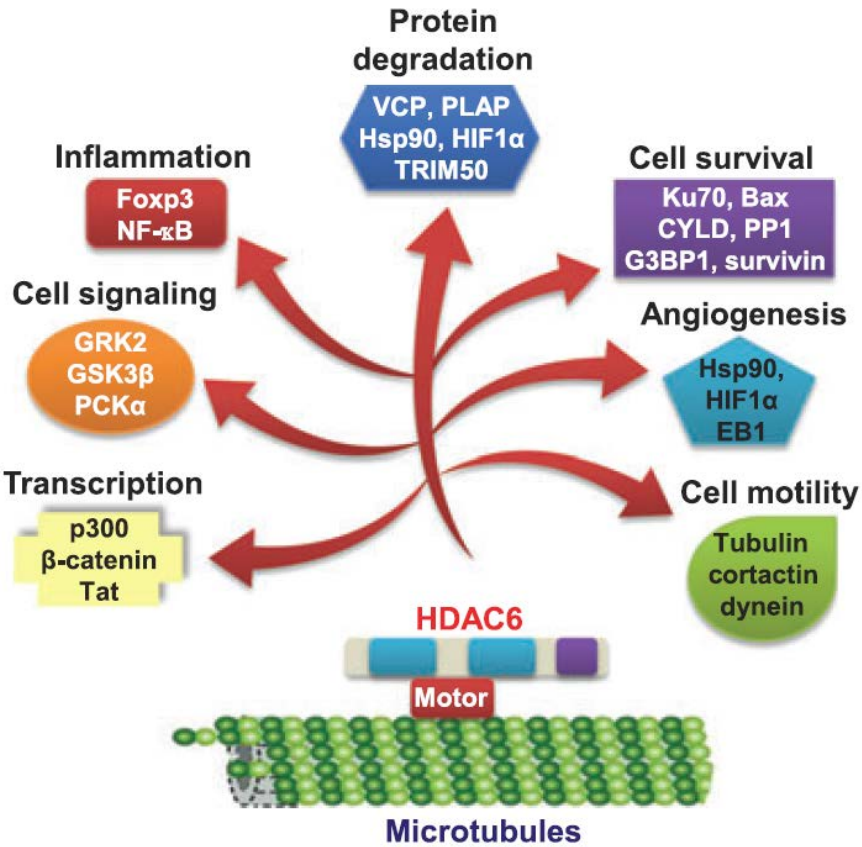


Figure 4. HDAC6 substrates and their functions (27)

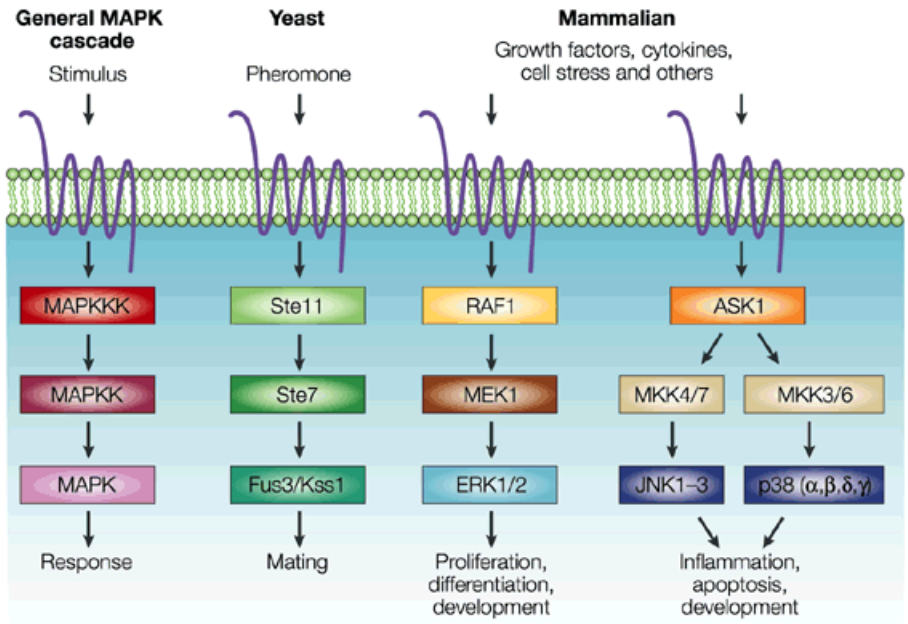


Figure 5. Mitogen-activated protein kinase (MAPK) cascades (40)

Purpose

This study aimed to investigate the effect of stress signal on the cell migration of non-small cell lung cancer cells and its underlying mechanisms.

The specific aim of this study was:

1. To investigate the effect of isoproterenol on the migration of lung cancer cells
2. To investigate the mechanism of isoproterenol to stimulate migration of lung cancer cells
3. To investigate the mechanism of isoproterenol to increase the expression of HDAC6 in lung cancer cells

Materials and Methods

1. Cell culture and reagents

The human non-small cell lung cancer H1299 and A549 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). These cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI 1640 containing 10% fetal bovine serum (FBS) (Welgene, Taegu, Korea) and 100 units/ml penicillin/streptomycin. The cells were cultured in a 5% CO_2 incubator at 37°C.

Forskolin and SP600125 were purchased from Calbiochem (CA, USA). SB203580 was purchased from Cayman (MI, USA). Actinomycin D, dimethyl sulfoxide (DMSO), Dulbecco's phosphate-buffered saline (DPBS), H89, isoproterenol (ISO), PD98056, PD0325901 and prostaglandin E_2 (PGE_2) were purchased from Sigma Chemicals (MO, USA). N6-phenyl-cAMP (6-Phe-cAMP) and 8- (4-cholorophenylthio)-2'-O-methyl-cAMP (8-pCPT-cAMP) were purchased from the Biolog Life Science Institute (Bremen, Germany).

2. Expression plasmids and transient transfection

The expression plasmid of the EE-tagged constitutively active mutant of Gαs (GαsQ227L) was purchased from the Missouri S&T cDNA Resource Center (MO, USA). The GαsQ277L mutant has a substitution of Leu-277 for Gln-277 that results in the inactivation of the intrinsic GTPase, which causes constitutive activation of the protein. Wild-type and dominant-negative CREBs (S133A, R287L) were gifts from Dr. Sahng-June Kwak (Dankook University, Cheonan, Korea). CARap1A/1B was kindly provided by Keith Burridge (University of North Carolina, USA) (41), the dominant negative PKA in MT-REVab was provided by Dr. G. Stanley McKnight (University of Washington, USA), and the catalytic subunit of PKA (GPKA) was provided by Dr. S.H. Green (42). The short hairpin RNAs (shRNAs) against HDAC6 and Rap1 and the control shRNA were purchased from Sigma Chemicals. Small interfering RNAs (siRNAs) against Epac1 and Epac2 were from Santa Cruz Biotechnology (CA, USA). Decoy oligonucleotides for the CRE (CRE decoy) were prepared as described previously (43). The H1299 cells were transfected with expression constructs and siRNAs using Lipofectamine 2000 (Invitrogen, CA, USA).

3. Quantitative reverse transcription polymerase chain reaction (qPCR)

The total RNA was isolated from the cells using the TRIzol reagent (Invitrogen). First-strand complementary DNA (cDNA) was synthesized using oligo-dT primers and the SuperScript II Reverse Transcriptase (Invitrogen). The specific oligonucleotide primers used in the qPCR were as follows: for HDAC6, 5'-TCAGGTCTACTGTGGTCGTT-3' and 5'-TCTTCACATCTAGGAGAGCC-3'; and for glyceraldehyde-3-phosphase dehydrogenase (GAPDH), 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'.

The qPCR was performed in a 20-μl mixture composed of forward and reverse primers (5 pmol) and SYBR Premix Ex Taq (Takara Bio Inc., Japan) using a C1000 thermal cycler (Bio-Rad, CA, USA). After 40 cycles of PCR, the average threshold cycle (Ct) values from triplicate qPCR experiments were normalized against the average Ct value of GAPDH.

4. Western blot analysis

The expressions of the proteins were analyzed by western blotting using specific antibodies. Antibodies against HDAC6 and Epac were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against HDAC4, acetylated tubulin, tubulin, and β-actin were from Sigma Chemicals. Antibodies against HDAC7, cAMP response element binding protein (CREB), phospho-CREB (Ser-133), c-Raf, phospho-c-Raf at Ser-338 and Ser-259, MEK1/2, phospho-MEK1/2, p44/42 MAPK, phospho-p44/42 MAPK, and Rap1A/1B were purchased from Cell Signaling Technology (MA, USA). The HDAC9 antibody was from Abcam (Cambridge, UK). The HDAC5 antibody was from Alexis Biochemicals (PA, USA). The horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Zymed (CA, USA) or Santa Cruz Biotechnology. The blots were incubated with an enhanced chemiluminescence substrate mixture (Pierce, Chester, UK), and the resulting blot images were then recorded with an LAS-3000 luminescent image analyzer system (Fuji, Tokyo, Japan). The densities of the protein bands were quantified using the Multi Gauge v3.0 software (Fuji), and the protein amounts are expressed as

multiples of the corresponding densities of the control.

5. Transwell migration assay

H1299 cells were transfected with shRNA targeting HDAC6 or scrambled sequences and incubated for 48 h. The cells (10,000) were then seeded onto the upper chamber (8-μm pore size) of a 24-well plate (Corning, Lowell, USA) containing DMEM, 1% FBS, and 20 μM isoproterenol. The lower chamber was filled with DMEM containing 10% FBS and 20 μM isoproterenol. After incubation for 16 h, the migrated cells were fixed and stained using Diff-Quik solution (Sysmex, Kobe, Japan), and the non-migrated cells were removed with a cotton swab. The cell numbers were counted in five different microscopic fields (Leica DFL 290, Wetzlar, Germany).

6. Assay of Rap1 activity

Rap1 activity was assayed by analyzing the binding of the activated proteins to the Rap1 binding domain (RBD) of the ral guanine nucleotide exchange factor (RalGDS) protein. The plasmid

encoding a GST fusion protein containing the 97-amino acid RBD of the RalGDS protein (pGEX RalGDS-RA) was a gift from J.H. Chung (NIH, USA)) (44). The cell lysates were incubated with GST-RalGDS RBD protein that was preincubated with glutathione-Sepharose 4B (GE Healthcare, USA) at 4 °C with agitation. The beads were washed three times with lysis buffer and then subjected to immunoblotting analysis.

7. Data analysis

All experiments were independently repeated at least three times, and the data are presented as the means ± the standard errors (SE). The non-parametric Mann-Whitney U-test was used to analyze the mean values, and P-values less than 0.05 were considered statistically significant.

Results

1. Isoproterenol signaling increases the expressions of HDAC6 protein and mRNA in H1299 lung cancer cells.

To examine the effects of stress signals on the expressions of HDACs, we treated H1299 human lung cancer cells with isoproterenol, which is an analog of the epinephrine stress hormone, and the expressions of HDACII family proteins were analyzed by western blotting. Treatment with isoproterenol increased the expression of HDAC6 without significantly altering the expressions of the other tested HDACII proteins ($p < 0.05$; Figure 6). Furthermore, treatment of the H1299 cells with another Gαs-coupled receptor agonist (prostaglandin E_2) or an adenylyl cyclase activator (forskolin) also increased HDAC6 expression (Figure 7). When the cells were treated with isoproterenol, the expression of HDAC6 mRNA increased at 6 h (Figure 8) and reached a plateau at 30 h, and the expression of the HDAC6 protein began to increase at 12 h (Figure 9). Dose dependent treatment manner of isoproterenol, 1 μM

to 100 μM of isoproterenol increased HDAC6 expression (Figure 10). To examine whether increased transcription might have caused the increase in HDAC6 expression, the effect of transcription inhibition via actinomycin D treatment on the isoproterenol-induced HDAC6 expression was assessed. Treatment with actinomycin D abolished the isoproterenol-induced increases in HDAC6 mRNA (Figure 11) and protein expressions (Figure 12). These results indicate that isoproterenol signaling increases HDAC6 protein expression by stimulating the transcription of the HDAC6 gene via cAMP signaling in lung cancer cells.

2. Isoproterenol decreases the acetylation of α-tubulin and increases the migration of H1299 lung cancer cells in an HDAC6-dependent manner.

HDAC6 deacetylates many non-histone proteins, including α-tubulin, and the acetylation of α-tubulin is involved in the regulation of cell migration. Thus, the effects of isoproterenol on the acetylation of α-tubulin and cell migration were examined. Treatment with isoproterenol significantly reduced the acetylation of α-tubulin at 36 h and 48 h after the treatment (Figure 13). When HDAC6 expression

was knocked down via transfection with HDAC6 shRNA, the acetylation of α-tubulin was increased (Figure 14). Treatment with isoproterenol increased the migration of the H1299 cells in the transwell migration assay, and this increase in migration was blocked by knockdown of HDAC6 with a specific shRNA (Figure 15). These results indicate that isoproterenol decreases the acetylation of α-tubulin and promotes cell migration by increasing HDAC6 expression in H1299 lung cancer cells.

3. Isoproterenol increases HDAC6 by inhibiting c-Raf-MEK-ERK pathways.

To probe the signaling pathway that mediated the HDAC6-increasing effect of isoproterenol signaling, the effects of mitogen-activated protein kinases (MAPKs) on HDAC6 expression were assessed following treatment with MAPK-specific inhibitors. Treatment with the extracellular signal-regulated kinase (ERK) inhibitor (PD98059) caused an increase in HDAC6 expression that was similar to that of isoproterenol treatment. However, treatments with a JNK inhibitor (SP600215) and a p38 inhibitor (SB203580) did not significantly alter the HDAC6 expression levels (Figure 16).

Treatment with PD0325901, another ERK inhibitor, also caused a significant increase in HDAC6 expression (Figure 17). Treatment with PD98059 increased the HDAC6 mRNA level, and the inhibition of transcription via actinomycin D abolished the effects of PD98059 on HDAC6 mRNA (Figure 18) and protein (Figure 19). Moreover, treatment with isoproterenol decreased the activating phosphorylation of ERK1/2, the upstream mitogen-activated extracellular signal-regulated kinase (MEK1/2), and c-Raf (S338) from 30 min until 4 h after the treatment (Figure 20). Furthermore, the expression of constitutively active MEK1 (caMEK1) abolished the isoproterenol-induced HDAC6 expression (Figure 21). Additionally, treatment of A549 cells, i.e., another human lung cancer cell line, with forskolin also inhibited the c-Raf-MEK1/2-ERK1/2 activity and increased the HDAC6 expression (Figure 22). These results indicate that isoproterenol increased HDAC6 expression via the inhibition of the c-Raf-MEK1/2-ERK1/2 signaling pathway in lung cancer cells.

4. Both the PKA and Epac pathways mediate isoproterenol-induced inhibitions of c-Raf.

To investigate the mechanism by which isoproterenol inhibited c-Raf-MEK1/2-ERK1/2 to increase HDAC6 expression, we analyzed the roles of the cAMP-dependent protein kinase (PKA) and exchange protein activated by cAMP (Epac) pathways, which are the two major signaling pathways that are activated by cAMP. The expression of a dominant negative PKA (dnPKA) did not block the isoproterenol-induced HDAC6 expression (Figure 23). Similarly, the knockdown of Epac1/2 with specific siRNAs did not block the isoproterenol-induced HDAC6 expression (Figure 24). However, the simultaneous transfection of dnPKA and Epac1/2 siRNAs abolished the isoproterenol-induced HDAC6 expression (Figure 25). The involvements of PKA and Epacs in HDAC6 expression were confirmed in a study utilizing selective agonists. Treatment with the PKA-selective agonist N6-phenyl-cAMP (6-Phe-cAMP) or the Epac selective agonist 8-pCPT-2'-O-Me-cAMP (8-pCPT-cAMP) decreased the activating phosphorylation of c-Raf at Ser-338 and increased the inhibitory phosphorylation of c-Raf at Ser-259. The inhibitions of c-Raf by these agonists resulted in decreased

phosphorylation of the downstream MEK1/2 and ERK1/2, which caused an increase in HDAC6 expression similar to that of isoproterenol treatment (Figure 26). Moreover, the expression of the catalytic subunit of PKA (GPKA) also induced HDAC6 expression (Figure 27). Additionally, the mediation of the 6-Phe-cAMP effect on HDAC6 expression by PKA was confirmed by the abolishment of the 6-Phe-cAMP effect by the expression of a dominant negative PKA (Figure 28). These results indicate that both the PKA and Epac pathways mediated the isoproterenol-induced inhibition of c-Raf and the increase in HDAC6 expression.

5. Rap1A mediates the Epac-induced inhibition of c-Raf.

To study how the Epac pathway inhibits c-Raf, we assessed the role of Rap1, which is a well-known downstream target of Epac. The effect of the Epac selective activator, 8-pCPT-cAMP, on HDAC6 expression was blocked by the knockdown of Epac with siRNA, which confirmed the selectivity of the agonist for Epac (Figure 29). Treatment with 8-pCPT-cAMP or isoproterenol increased the amount of Rap1 bound to Rap1 binding domain of the RalGDS protein in a pull-down assay (Figure 30). Because Rap1 has two isoforms,

i.e., Rap1A and Rap1B, we determined which isoform mediated the HDAC6-expressing effect by transfecting each of the constitutively active forms of Rap1 into the H1299 cells. The H1299 cells that expressed the constitutively active Rap1A (caRap1A) exhibited increased HDAC6 expression and decreased phosphorylations of c-Raf, MEK1/2 and ERK1/2, but the cells that expressed the constitutively active Rap1B (caRap1B) did not exhibited increased HDAC6 expression or decreased phosphorylation of c-Raf (Figure 31). The knockdown of Rap1A with shRNA abolished the 8-pCPT-cAMP-induced HDAC6 expression, which confirmed the mediating role of Rap1A (Figure 32). These results indicate that Epac increased HDAC6 expression by activating Rap1A.

To study whether ERK mediates isoproterenol-induced HDAC6 expression via CREB, a well-known transcription factor that is activated by PKA, the effect of CREB inhibition by transfecting dominant negative CREBs or CRE decoy oligonucleotides on PD98059-induced expression of HDAC6 were analyzed. The PD98059-induced increase in HDAC6 expression was not abolished by the transfection dominant negative CREBs (S133A and R287L) or CRE decoy oligonucleotides (Figure 33 & 34). CREB inhibition by the

dominant negative CREB (S133A) or CRE decoy oligonucleotides also abolished the increase in HDAC6 expression by isoproterenol treatment (Figure 35). These results suggest that isoproterenol increases HDAC6 expression in a CREB-independent pathway.

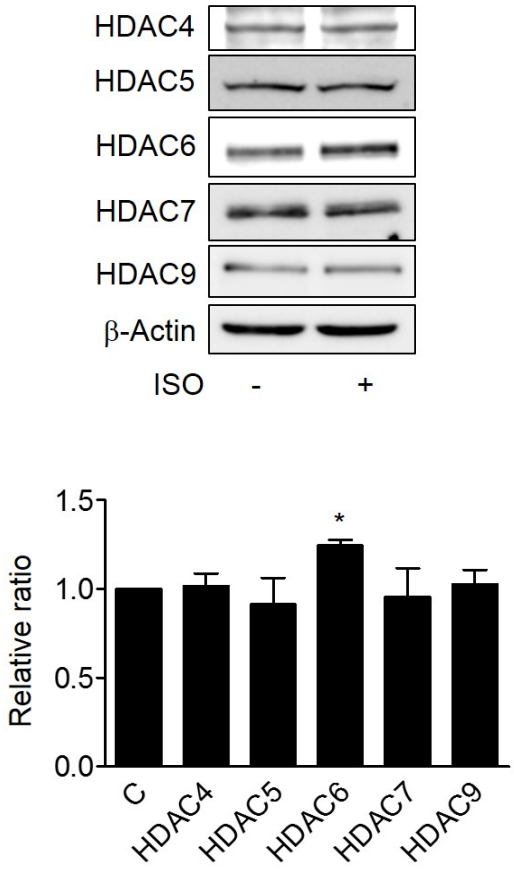


Figure 6. Effects of isoproterenol on the expression of type II HDACs in H1299 human lung cancer cells.

H1299 human lung cancer cells were treated with 20 μM isoproterenol (ISO) and harvested at 48 h for western blot analysis. β-Actin was analyzed as loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

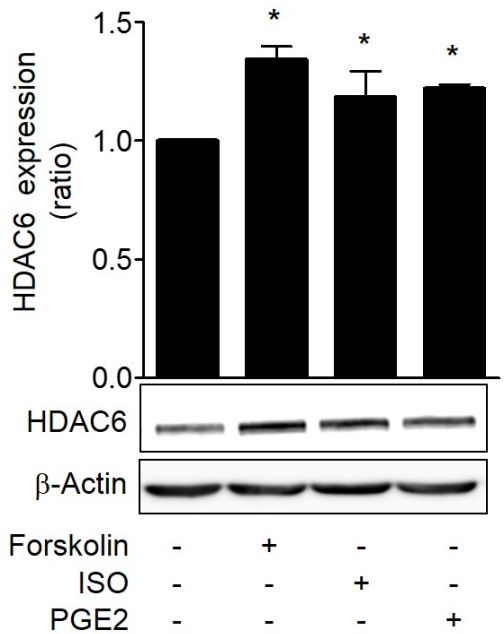


Figure 7. Effects of cAMP signaling on the expression of HDAC6.

H1299 human lung cancer cells were treated with 20 μM isoproterenol (ISO), 20 μM forskolin or 20 μM PGE_2 and harvested at 48 h for western blot analysis. β-Actin was analyzed as loading control. Western band density was quantified using Multi Gauge v.2.3 software and expressed as a ratio relative to the control band density. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

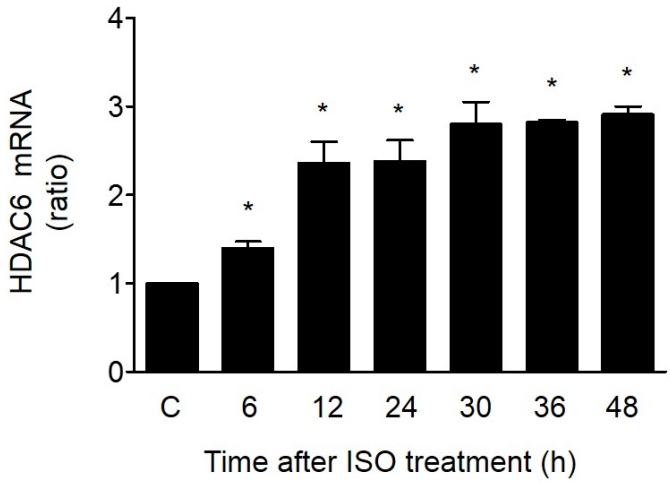


Figure 8. Temporal patterns of the expression of HDAC6 mRNA following isoproterenol treatment.

H1299 cells were treated with 20 μM isoproterenol and harvested at the indicated time points. The HDAC6 mRNA level was analyzed by quantitative real time PCR, normalized to GAPDH mRNA, and expressed as a ratio relative to the control level. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

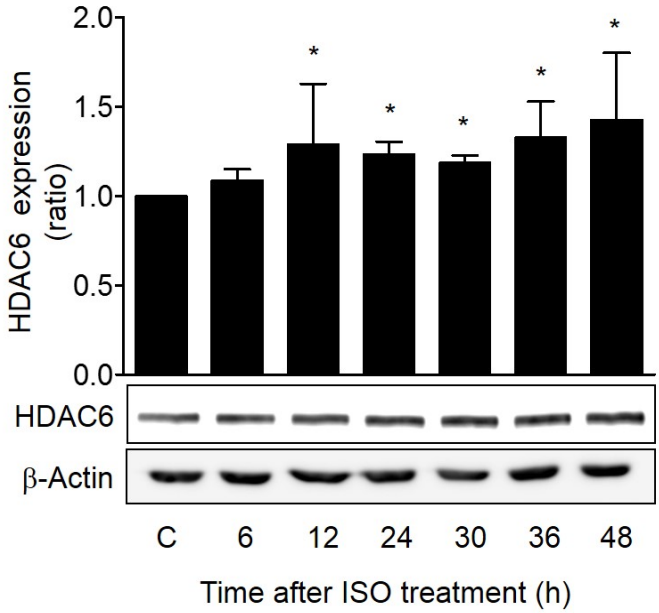


Figure 9. Temporal patterns of the expression of HDAC6 protein following isoproterenol treatment.

H1299 cells were treated with 20 μM isoproterenol and harvested at the indicated time points for western blot. β-Actin was analyzed as a loading control. The western blot band densities were quantified using Multi Gauge v.2.3 software and are expressed as ratio relative to the control band density. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

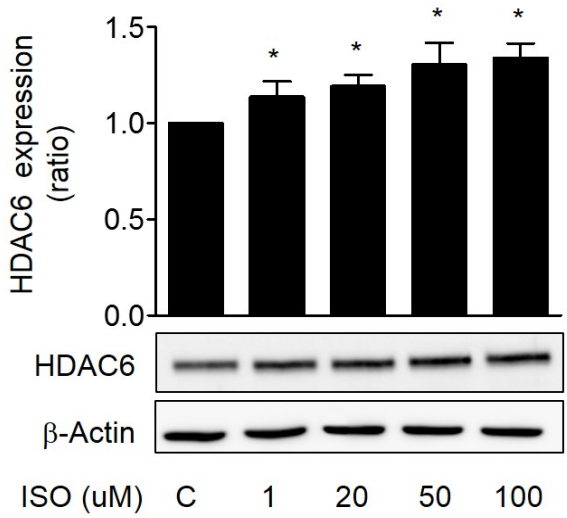


Figure 10. Dose dependent manner of the expression of HDAC6 protein following isoproterenol treatment.

H1299 cells were treated with 1-100 μM isoproterenol and harvested at 48 h for western blot analysis. β-Actin was analyzed as loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

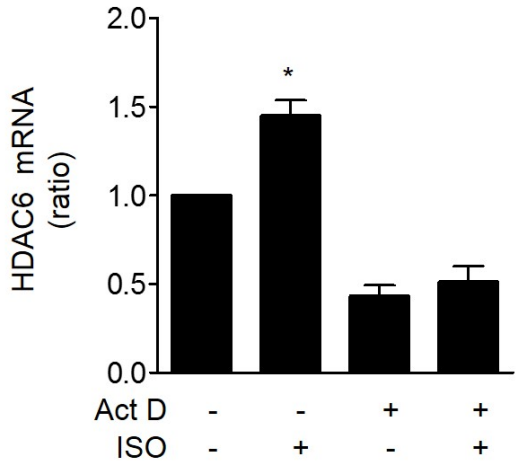


Figure 11. Effects of transcription inhibition on the isoproterenol-induced increase in HDAC6 RNA expression.

H1299 cells were pre-treated with 8 μM actinomycin D for 2 h before isoproterenol treatment (20 μM). The cells were harvested at 30 h for analysis of mRNA levels. The HDAC6 mRNA level was analyzed by quantitative real time PCR, normalized to GAPDH mRNA, and expressed as a ratio relative to the control level. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

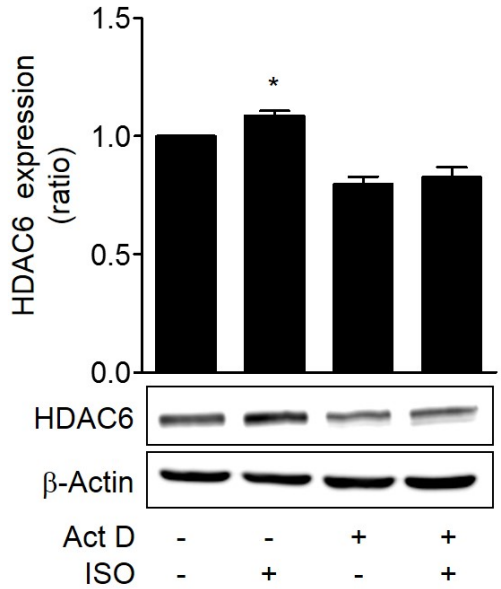


Figure 12. Effects of transcription inhibition on the isoproterenol-induced increase in HDAC6 protein expression.

H1299 cells were pre-treated with 8 μM actinomycin D for 2 h before isoproterenol treatment (20 μM). The cells were harvested at 48 h for western blot analysis. β-Actin was analyzed as a loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

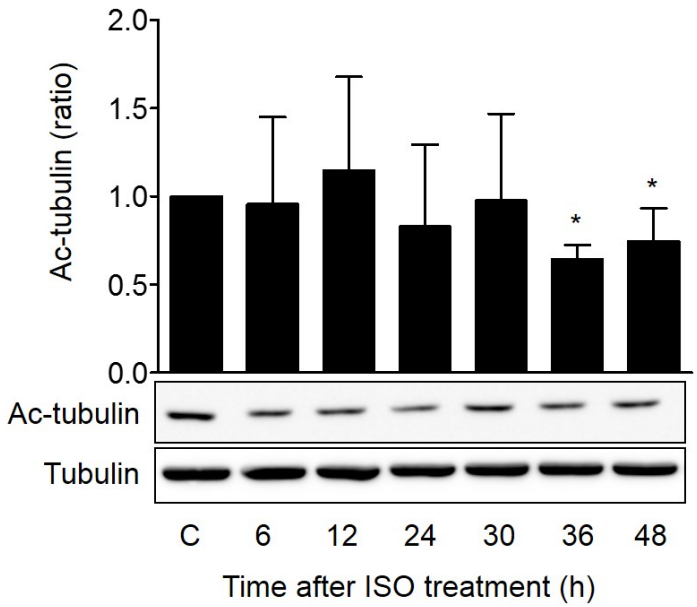


Figure 13. Effects of isoproterenol on the acetylation of α-tubulin.

H1299 cells were treated with 20 μM isoproterenol, and the levels of acetylated α-tubulin (Ac-tubulin) and total tubulin were assessed at the indicated time points by western blotting. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

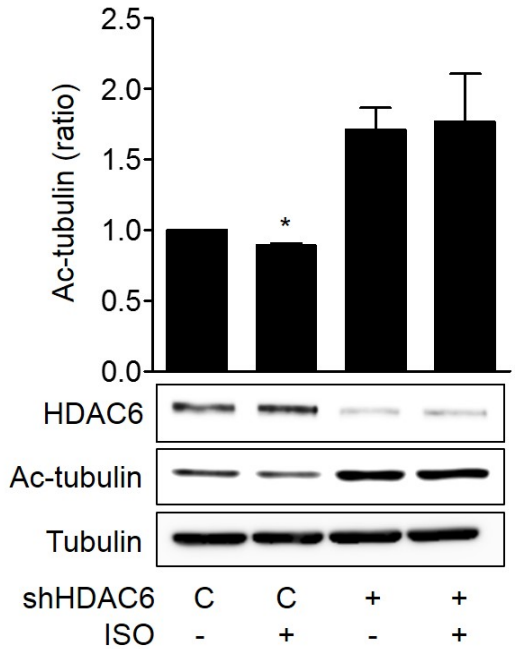
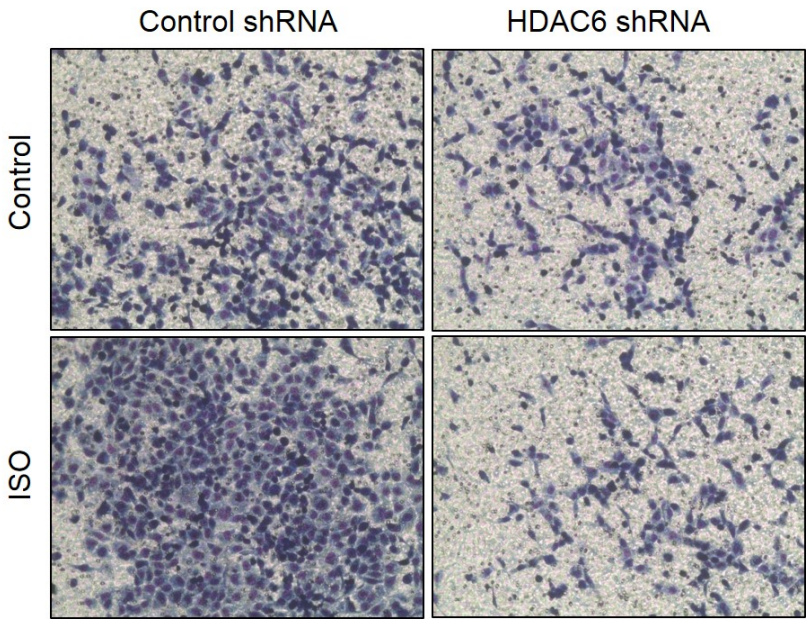


Figure 14. Effects of HDAC6 knock down on the isoproterenol-induced deacetylation of α-tubulin.

H1299 cells in 100 mm dishes were transfected with shRNA targeting HDAC6 or control shRNA (10 μg) and incubated for 24 h. Then, the cells were treated with 20 μM isoproterenol for an additional 48 h prior to harvesting for western blot analysis. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).


Control shRNA
HDAC6 shRNA
Control
ISO

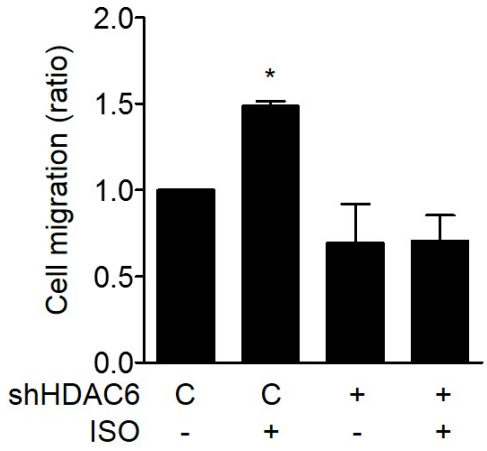

Cell migration (ratio)
2.0
1.5
1.0
0.5
0.0
*
shHDAC6 C C + +
ISO - + - +

Figure 15. Effects of isoproterenol on the migration of H1299 lung cancer cells.

A transwell migration assay was performed. In brief, H1299 cells were transfected with shRNA targeting HDAC6 or control shRNA, and the cells were seeded onto the upper chamber containing 20 μM isoproterenol. After 16 h, the migrated cells were fixed and stained, and representative photographs (100x magnification) of the migrated cells are presented. The migrated cells were counted in five different microscopic fields, and the averages were calculated. The values presented are the means ± the SDs of three independent experiments. Asterisks (*) indicate significant differences from the isoproterenol-untreated control ($P<0.05$, Mann-Whitney *U*-test).

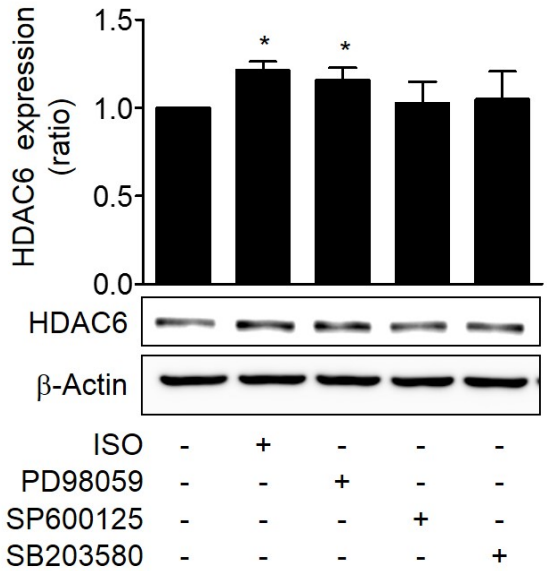


Figure 16. Effects of MAPK inhibitors on HDAC6 expression.

H1299 cells were treated with 20 μM isoproterenol (ISO), 20 μM PD98059, 10 μM SP600215, or 20 μM SB03580 for 48 h then HDAC6 expression was analyzed by western blotting and β-Actin was analyzed as a loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

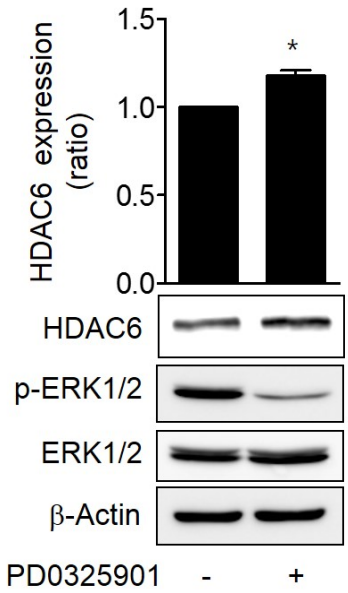


Figure 17. Effect of PD0325901 on HDAC6 expression in H1299 cells.

H1299 cells were treated with 1 μM PD0325901 for 48 h then HDAC6 expression was analyzed. β-Actin was analyzed as a loading control for western blot. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

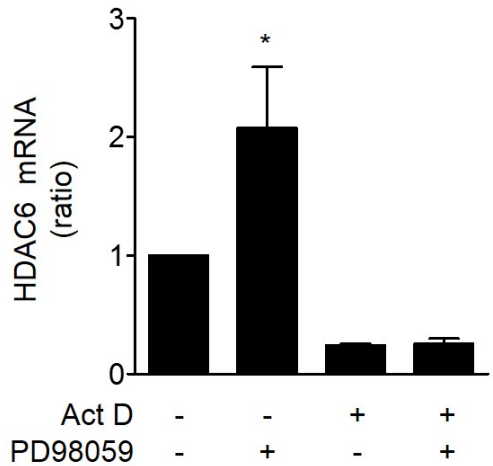


Figure 18. Effect of transcription inhibition on MEK inhibitor-induced increase in HDAC6 RNA.

H1299 cells were pre-treated with 40 nM actinomycin D for 2 h, then PD98059 was treated for 30 h mRNA was assessed. The HDAC6 mRNA level was analyzed by quantitative real time PCR, normalized to GAPDH mRNA, and expressed as a ratio relative to the control level. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

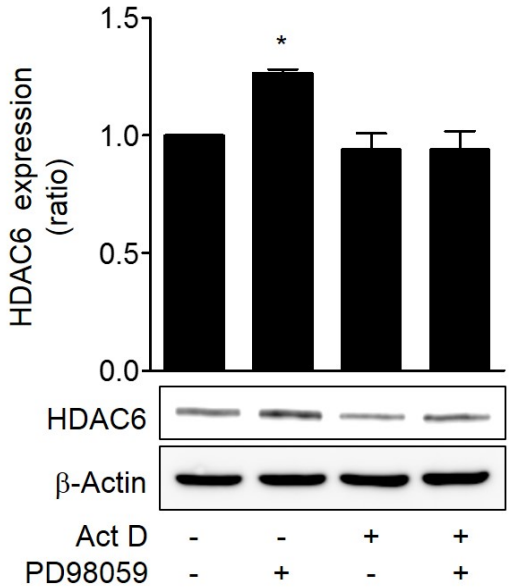


Figure 19. Effect of transcription inhibition on MEK inhibitor-induced increase in HDAC6 protein.

H1299 cells were pre-treated with 40 nM actinomycin D for 2 h, then PD98059 was treated for 46 h then HDAC6 expression was analyzed by western blot. β-Actin was analyzed as a loading control. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

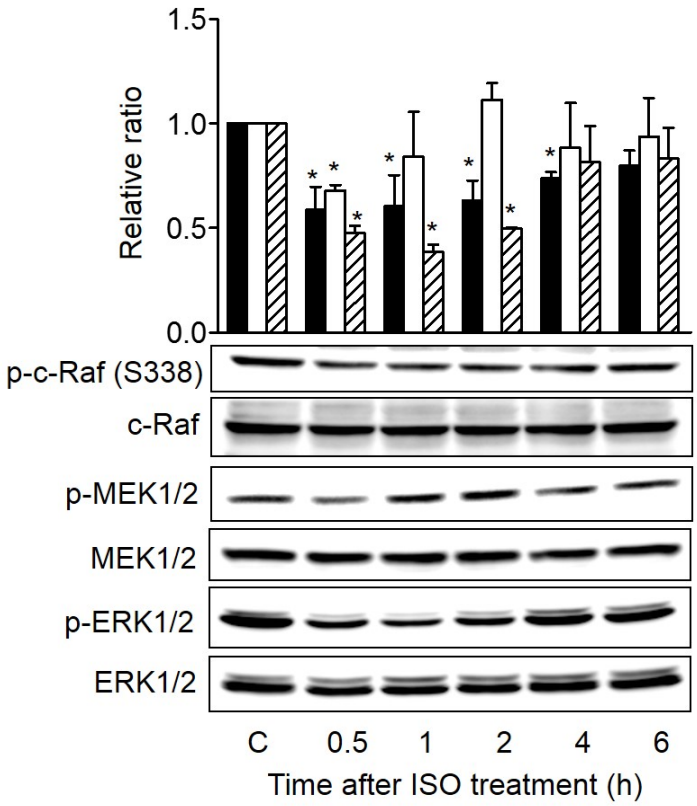


Figure 20. Effects of isoproterenol on c-Raf-MEK-ERK signaling.

H1299 cells were treated with isoproterenol for the indicated times, and the phosphorylations of c-Raf (filled bar), MEK (empty bar), and ERK (slant bar) were then analyzed by western blotting and densitometry. An asterisk indicates a statistically significant difference compared with the respective control (*, $p < 0.05$; Mann-Whitney *U*-test).

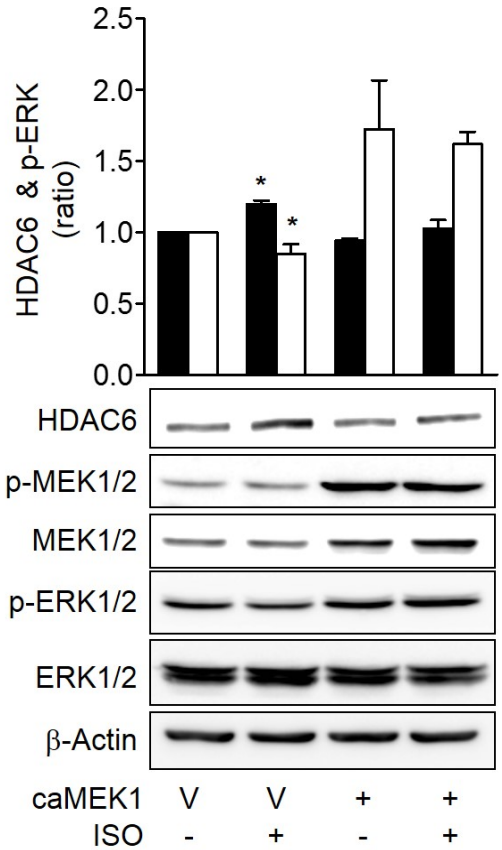


Figure 21. Effects of ERK activation on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with constitutively active MEK1 (caMEK1) for 24 h and were then treated with isoproterenol for 48 h prior to analysis. β-Actin was analyzed as a loading control. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the isoproterenol-untreated control ($P<0.05$, Mann-Whitney *U*-test).

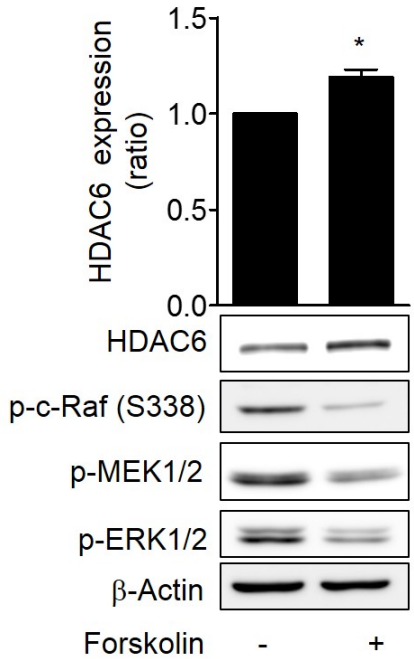


Figure 22. Effect of forskolin on HDAC6 expression and c-Raf-MEK-ERK pathway in A549 lung cancer cells.

A549 cells were treated with forskolin (20 μM) before HDAC6 expression was analyzed. β-actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

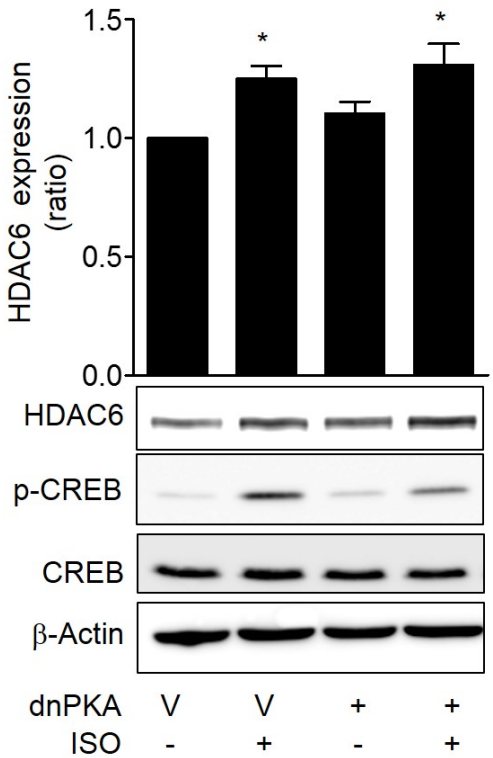


Figure 23. Effects of PKA inhibition on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with dominant negative PKA (dnPKA) for 24 h then, treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

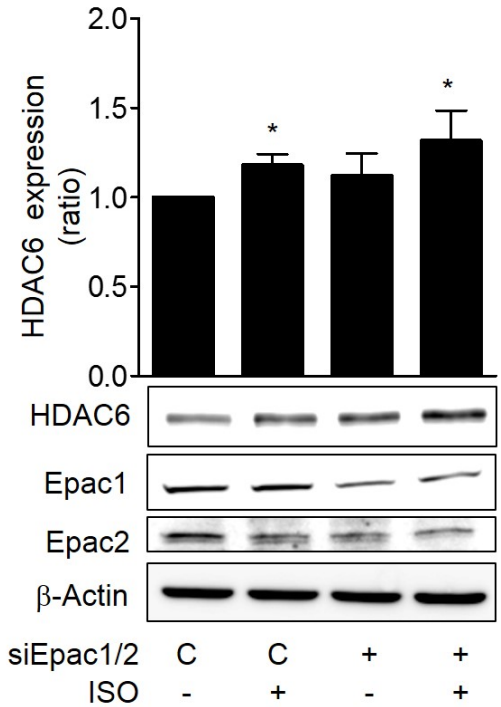


Figure 24. Effects of Epac knock down on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with Epac1 siRNA, Epac2 siRNA, scrambled control siRNA, incubated for 24 h, and then treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

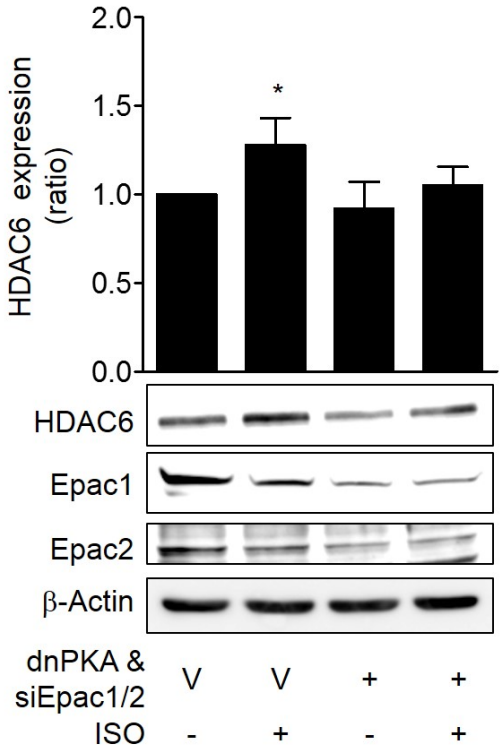


Figure 25. Effect of simultaneous PKA inhibition and Epac knock down on the isoproterenol-induced increase in HDAC6 expression.

H1299 cells were transfected with dominant negative PKA (dnPKA), Epac1siRNA, Epac2 siRNA, scrambled control siRNA, incubated for 24 h, and then treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

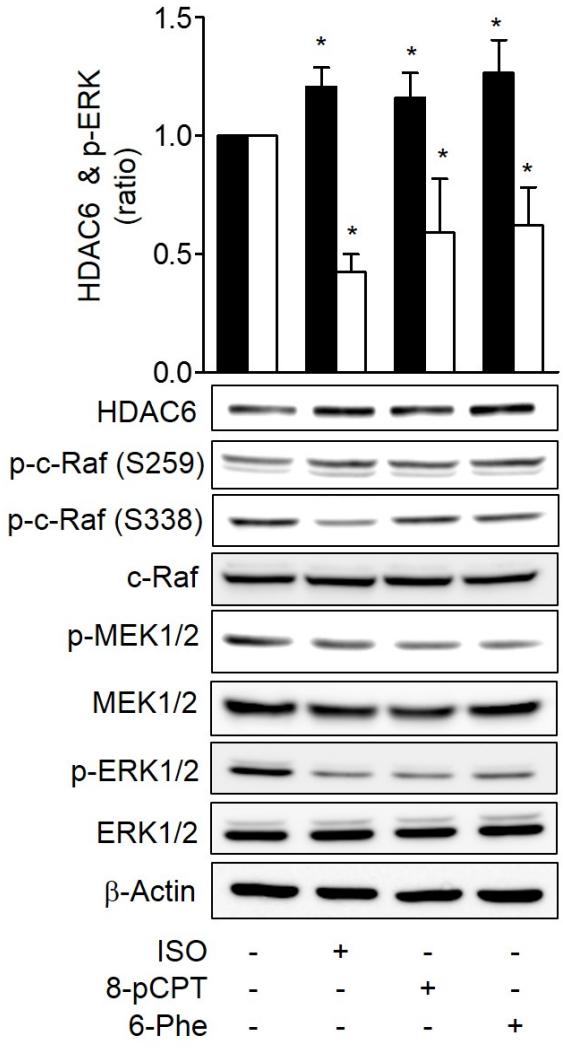

HDAC6 & p-ERK (ratio)
1.5
1.0
0.5
0.0
*
HDAC6
p-c-Raf (S259)
p-c-Raf (S338)
c-Raf
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
β-Actin
ISO - + - -
8-pCPT - - + -
6-Phe - - - +

Figure 26. Effects of Epac- and PKA-selective agonists on HDAC6 expression and c-Raf-MEK-ERK pathways.

H1299 cells were treated for 48 h with 20 μM ISO, 20 μM 8-pCPT-2' o-Me-cAMP (8-pCPT), EPAC activator, or 30 μM N6-Phenyl-cAMP (6-Phe) for 48 h before western blot analysis. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

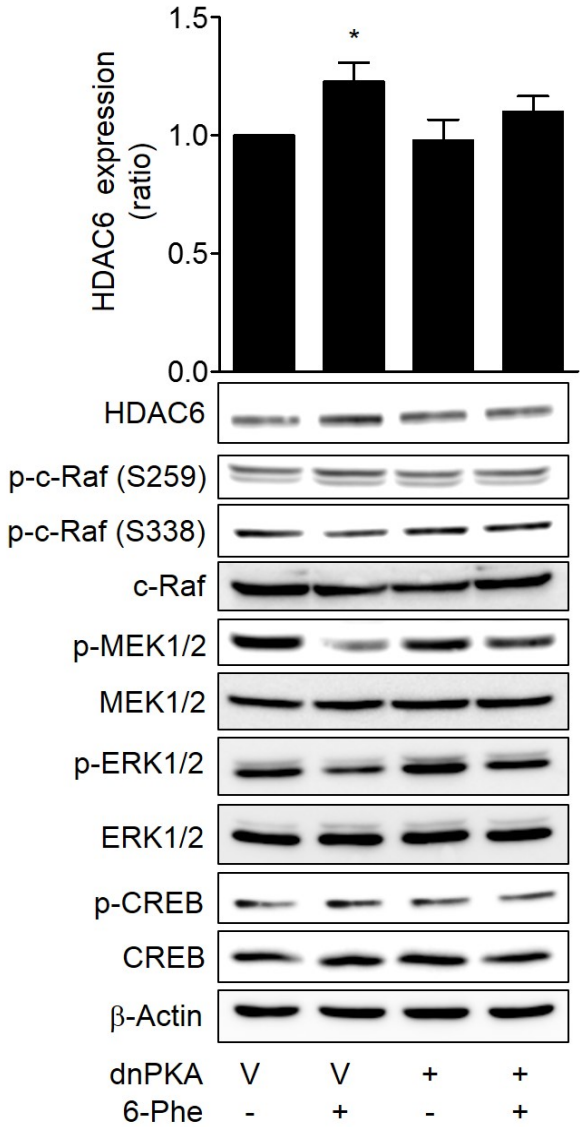

1.5
1.0
0.5
0.0
HDAC6 expression (ratio)
*
HDAC6
p-c-Raf (S259)
p-c-Raf (S338)
c-Raf
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
p-CREB
CREB
β-Actin
dnPKA V V + +
6-Phe - + - +

Figure 27. Effect of PKA inhibition on 6-Phe-induced HDAC6 expression and inhibition of c-Raf-MEK-ERK pathway.

H1299 cells were transfected with dominant negative PKA (dnPKA) for 24h then, treated with 30 μM N6-phenyl-cAMP (6-Phe) for 48 h. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

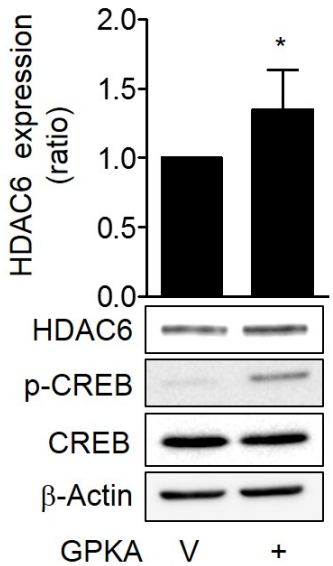


Figure 28. Effects of PKA activation on HDAC6 expression.

H1299 cells were transfected with the catalytic subunit of PKA (GPKA) or EGFP vector for 48 h. Then HDAC6 expression was analyzed by western blot. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

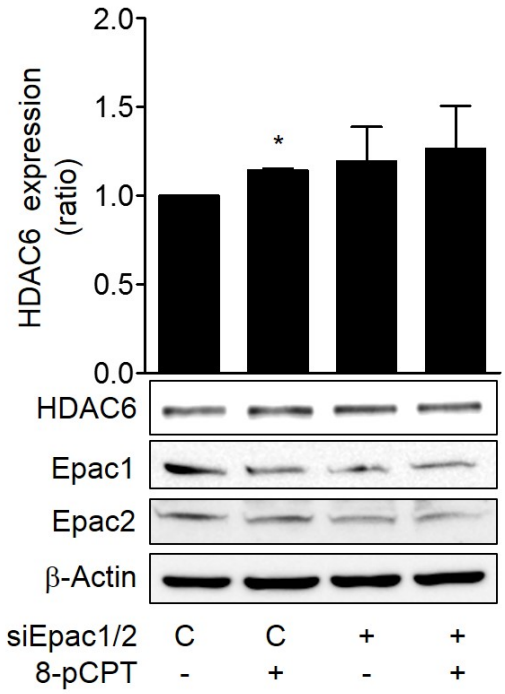


Figure 29. Effects of Epac knock down on 8-pCPT-cAMP-induced increase in HDAC6 expression.

Epac1 and 2 or control siRNA were transfected for 24 h. Then, 20 μM 8-pCPT-cAMP treatment was applied for 48 h prior to western blot analysis. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

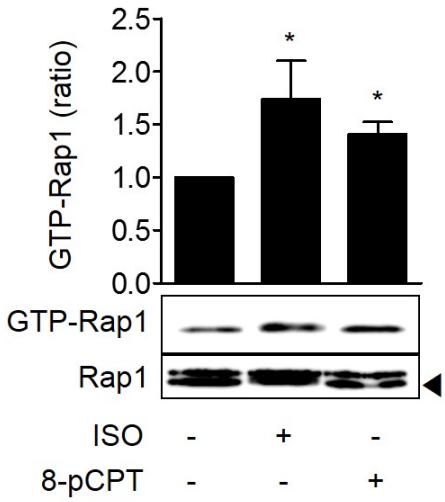


Figure 30. Effects of isoproterenol on Rap1 activation.

H1299 cells were treated with 20 μM isoproterenol or 20 μM 8-pCPT-cAMP for 30 min. The GTP-bound Rap1 was then pulled down using the immobilized Rap1-binding domain (RBD) of RalGDS, and western blot analysis was subsequently performed. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

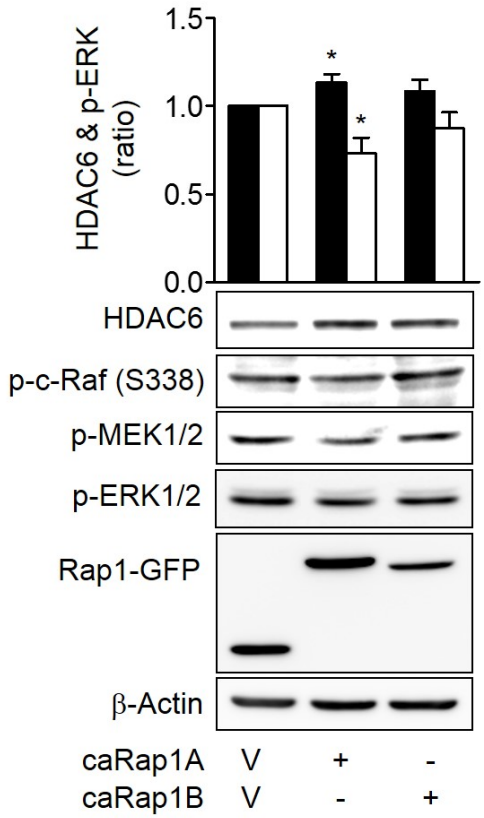

HDAC6 & p-ERK (ratio)
1.5
1.0
0.5
0.0
*
*
HDAC6
p-c-Raf (S338)
p-MEK1/2
p-ERK1/2
Rap1-GFP
β-Actin
caRap1A V + -
caRap1B V - +

Figure 31. Effects of Rap1 activation on ERK phosphorylation and HDAC6 expression.

H1299 cells were transfected with constitutively active Rap1A (caRap1A) and Rap1B (caRap1B), or EGFP control constructs (V) and incubated for 48 h prior western blot analysis. β-Actin was analyzed as a loading control for western blot. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$, Mann-Whitney *U*-test).

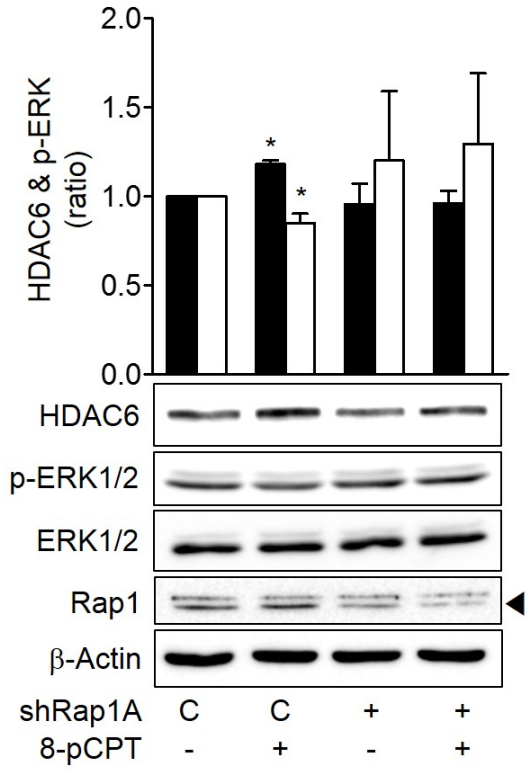


Figure 32. Effects of Rap1A knock down on 8-pCPT-cAMP-induced ERK phosphorylation and HDAC6 expression.

H1299 cells were transfected with Rap1A shRNA, incubated for 24 h, and then treated with 20 μM 8-pCPT-cAMP for 48 h. β-Actin was analyzed as a loading control for western blot. Filled bar represents HDAC6 expression and empty bar represents p-ERK. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

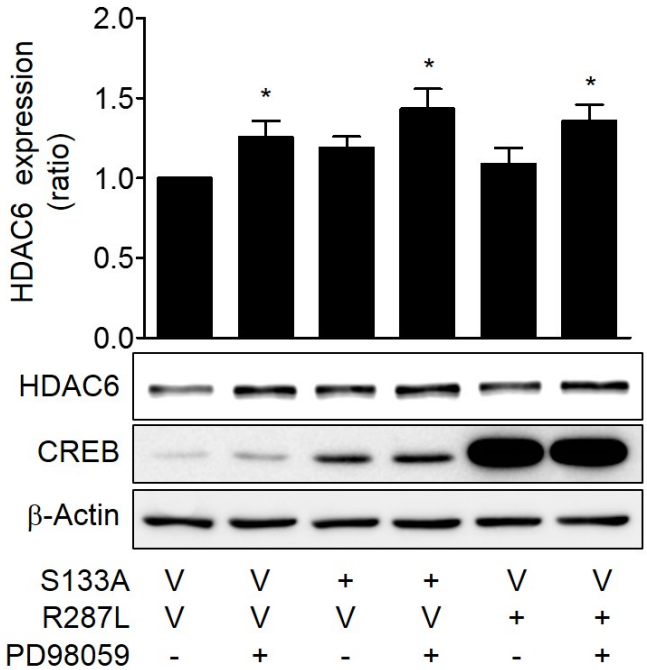


Figure 33. Effects of dominant negative CREB on PD98059-induced HDAC6 expression.

H1299 cells were transfected with dominant-negative CREBs (S133A, R287L), respective control vectors (V) for 24 h then 20 μM PD 98059 was treated for 48 h. Then HDAC6 expression was analyzed by western blot. β-Actin was analyzed as a loading control for western blot. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

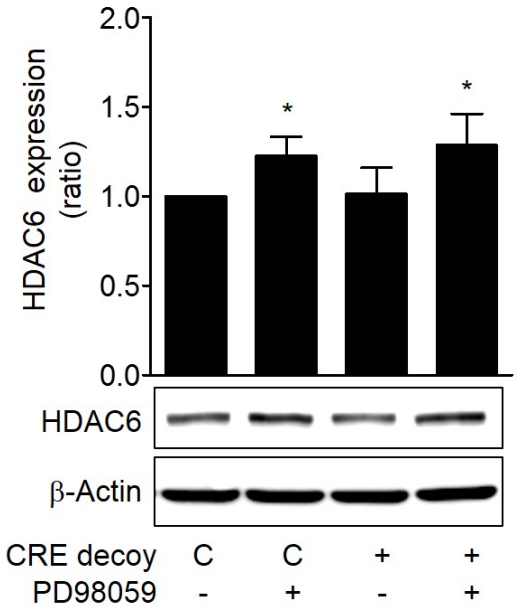


Figure 34. Effect of inhibition of CREB binding to CRE on PD98059 induced HDAC6 expression.

H1299 cells were transfected with oligonucleotides (CRE decoy or CRE control (C)) for 24 h then PD 98059 was treated for 48 h western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

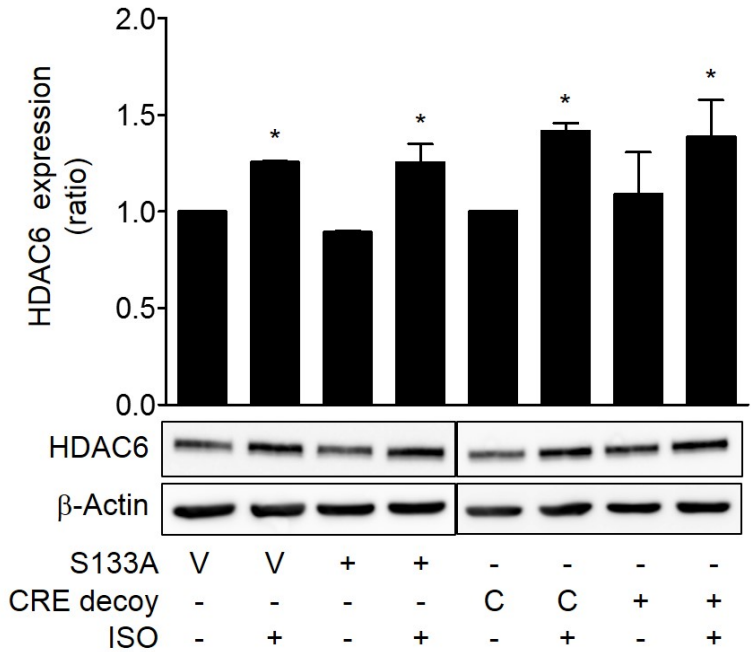


Figure 35. Effects of CRE decoy oligonucleotides CREB on isoproterenol-induced HDAC6 expression.

H1299 cells were transfected with dominant-negative CREBs (S133A, R287L), respective control vectors (V), or CRE decoy and control (C) oligonucleotides and then incubated for 24 h. The cells were then treated with 20 μM isoproterenol (ISO) for 48 h before western blot analysis. β-Actin was analyzed as a loading control. Asterisks (*) indicate significant differences from the respective control cells ($P<0.05$ Mann-Whitney *U*-test).

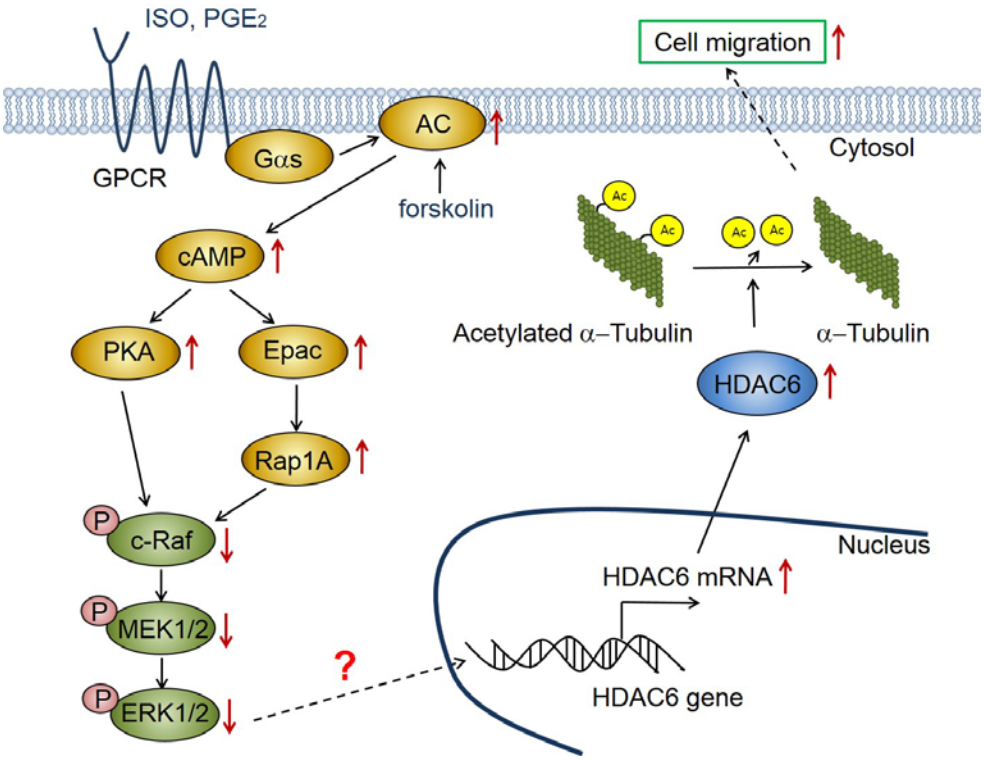


Figure 36. A suggested mechanism by which isoproterenol increases HDAC6 expression in human lung cancer cells.

Isoproterenol increases HDAC6 expression via the PKA- and Epac-mediated inhibitions of c-Raf-MEK-ERK, which result in increased cell migration. The solid lines indicate proven signaling pathways, and the dotted lines indicate potential signaling pathways. GPCR, G-protein-coupled receptor.

Discussion

This study investigated the effects of stress signals on HDAC expression and their underlying mechanisms in human lung cancer cells. We found that isoproterenol signaling increases HDAC6 expression by inducing transcription through a PKA/Epac/ERK-dependent pathway and that the isoproterenol-induced increase in HDAC6 expression stimulates the migration of H1299 lung cancer cells (Figure 36).

Our finding that isoproterenol signaling increased the expression of HDAC6, which stimulated the migration of lung cancer cells, is supported by the result that treatment with isoproterenol increased the expressions of HDAC6 mRNA and protein without affecting the expressions of other class II HDACs in H1299 and A549 human lung cancer cells and that isoproterenol treatment caused a decrease in the acetylation of α-tubulin and an increase in cell migration in an HDAC6-dependent manner. Isoproterenol is an isopropyl analog of epinephrine, which is a major stress hormone, and activates β-adrenergic receptors, which are the receptors for

epinephrine and norepinephrine. Therefore, our findings suggest that stress might increase HDAC6 expression, which might in turn stimulate the migration of cancer cells. HDAC6 has been reported to deacetylate Hsp90 to regulate glucocorticoid receptor chaperone dynamics in the brain, which provides a promising strategy to reduce the harmful socioaffective effects of stress and glucocorticoids (36, 37). Chronic social stress has been found to decrease the expression of histone deacetylase 2 in the mouse brain (35). However, to the best of our knowledge, this is the first report to reveal that the isoproterenol stress signal increases HDAC6 expression in lung cancer cells.

HDAC6 is predominantly localized in the cytoplasm and regulates many important biological processes, including cell migration, immune synapse formation, transcription, cell proliferation and death, and the degradation of misfolded proteins (45). HDAC6 deacetylates α-tubulin and thus increases microtubule dynamics to increase cell motility (46), and it also modulates actin-dependent cell movement by altering the acetylation status of cortactin (47). Thus, we suggest that the increase in HDAC6 expression by isoproterenol stimulate the migration of lung cancer cells via the deacetylations of

α-tubulin and cortactin. A similar role of HDAC6 on cell migration and invasion has been reported in hepatocellular carcinomas (48). Stress signals, such as norepinephrine, have been reported to enhance invasive potential via the up-regulation of matrix metalloproteinases in ovarian and prostate cancer cells (3, 4). Our paper presents a novel mechanism, i.e., the up-regulation of HDAC6 expression, by which stress signals might stimulate the invasiveness of cancer cells. In addition, HDAC6 has been reported to deacetylate Hsp90 to regulate glucocorticoid receptor chaperone dynamics in the brain, which provides a promising strategy to reduce the harmful socioaffective effects of stress and glucocorticoid (36, 37). Furthermore, because HDAC6 are known to interact a variety of proteins (49), isoproterenol-induced HDAC6 expressions could regulate the other biological responses involved in cancer progression. HDAC6 can shuttle into the nucleus and deacetylate histone in vitro and in vivo, and therefore the effect of isoproterenol-induced HDAC6 expression on epigenetic regulation need to be further investigated.

We found that isoproterenol increases HDAC6 by activating a cAMP signaling pathway that involves both PKA and Epac, both of

which inhibit the c-Raf-MEK-ERK pathway in lung cancer cells. This finding is based on the result that HDAC6 expression was increased when cAMP signaling was activated by the expression of constitutively active Gαs or treatment with Gαs-coupled receptor agonists (i.e., PGE_2 and isoproterenol) and an adenylyl cyclase activator (forskolin) in the H1299 and A549 lung cancer cells. Similar to epinephrine and norepinephrine, isoproterenol binds β-adrenergic receptors to trigger the sequential activation of stimulatory G proteins, adenylyl cyclases, PKA, and Epac signaling (50). This finding suggests that HDAC6 expression might be increased not only by the stress signal isoproterenol but also by other signals that increase cAMP concentrations, such as phthalates (51). Additionally, cAMP signaling has been reported to stimulate HDAC4 activity in macrophages (52) and to decrease sirtuin 6 expression in lung cancer cells (53). Thus, we suggest that stress signals might regulate histone acetylation and gene expression by various ways via activating cAMP signaling.

cAMP signaling regulates various cellular responses by activating three major cAMP effector molecules: cAMP-dependent protein kinase (PKA), exchange factor directly activated by cAMP

(Epac), and cyclic-nucleotide-gated ion channels (CNG) (54). This study revealed that both PKA and Epac mediate the HDAC6-increasing effect of isoproterenol by demonstrating that the inhibition of either PKA or Epac alone did not abolish the effect of isoproterenol on HDAC expression, but the simultaneous inhibition of both PKA and Epac abolished this effect. Furthermore, treatment with either a PKA- or Epac-selective agonist increased HDAC6 expression. The involvement of PKA was further evidenced by the demonstration that the expression of the PKA catalytic subunit increased HDAC6 expression, and Epac-selective agonist elicited increased HDAC6 expression in via a Rap1A-dependent pathway.

In the study on the mechanisms by which PKA and Epac increased HDA6 expression, the PKA and Epac pathways were found to mediate the HADC6-increasing effect of isoproterenol by inhibiting c-Raf-MEK-ERK signaling. This finding is supported by the results that treatment with isoproterenol and selective agonists of PKA or Epac together inhibited the c-Raf-MEK-ERK signaling pathway, the inhibition of the c-Raf-MEK-ERK signaling pathway increased the HDAC6 expression, and isoproterenol increased HDAC6 expression in an ERK inhibition-dependent manner. ERK is

a member of the mitogen-activated protein kinase (MAPK) family and participates in the regulation of various processes, including cell migration and proliferation and transcription. ERK is activated by following the classical cascade of consecutive activating phosphorylation events: Raf phosphorylates and activates MEK, and activated MEK phosphorylates and activates ERK (55). The cAMP and MAPK pathways do not act independently; rather, multiple forms of cross-talk between these pathways can occur (56). As shown in our paper, cAMP signaling has previously been reported to inhibit the c-Raf-MEK-ERK pathway by decreasing activating phosphorylation at Ser-338 and increasing inactivating phosphorylation at Ser-259 on the c-Raf protein (27, 57, 58). The Rap1 protein also inhibits c-Raf activation via the sequestration of c-Raf from Ras through competition for Ras (59, 60).

In the present study, we found that isoproterenol increases HDAC6 expression by inhibiting the c-Raf-MEK-ERK pathway, but the mechanism by which the inhibition of ERK induces HDAC6 expression in a CREB-independent pathways requires elucidation. Phthalates have been reported to increase HDAC6 expression via PKA-dependent CREB phosphorylation, which results in increased

CREB binding to a CRE site of the HDAC6 promoter region (51). However, the increase in HDAC6 expression induced by isoproterenol or PD98059 was not abolished by blocking the binding of active CREB to CRE sites using dominant negative CREBs and CRE decoy oligonucleotides in our study, which suggests other mechanisms in isoproterenol-induced increase in HDAC6 expression in lung cancer cells. The ETS transcription factor Erg has been reported to regulate the expression of HDAC6 in human vascular endothelial cells (61), and Erg has domains that act as docking platforms for mitogen-activated protein (MAP) kinases; such docking leads to phosphorylation and the enhancement of transactivation activity (62). Thus, we speculated that ERK signaling might inhibit Erg activation or induce the expression of genes that repress HDAC6 gene transcription.

Conclusion

The following conclusions were obtained from this study.

1. Isoproterenol increases the migration of H1299 non-small cell lung cancer cells by HDAC6-induced decrease in acetylation of α-tubulin.

2. Isoproterenol stress signaling increases HDAC6 expression via a PKA/Epac/ERK-dependent pathway.

This study suggests that stress signals may stimulate the migration of cancer cells by increasing the expression of HDAC6.

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국문 초록

임정아
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스트레스는 암의 성장, 진행, 전이와 관련 있다. 에피네프린 신호는 스트레스 상태에서 활성화된다. 에피네프린의 아날로그인 이소프로테레롤은 베타 아드레날린 수용체와 결합하여 활성화시켜 cAMP 산물을 촉진시키고, 다양한 세포의 대사와 유전자 발현을 조절한다. 알파 튜불린은 히스톤 탈아세틸화효소 6 (HDAC6)에 의해 탈아세틸화되며, 미세소관의 역학에 영향을 주어 세포의 운동성을 조절한다. 이 연구에서는 스트레스 신호가 비소세포성 폐암 세포주에서 HDAC6의 발현 조절을 통해 세포의 이동에 영향을 주는 기전을 확인하고자 한다. H1299 폐암 세포주에 이소프로테레놀을 처리하였을 때 알파 튜불린의 아세틸화를 감소하였으며, 세포의 운동성을 촉진되었다. 또한 이소프로테레놀의 처리는 HDAC6의 발현이 증가시켰다. 이소프로테레놀에 의해 활성화된 세포의 이동은 HDAC6 를 감소시켰을 때 억제되었다. Protein kinase A (PKA) 활성제인 N6-phenyl-cAMP 나 exchange protein activated by cAMP (Epac)의 활성제인 8-pCPT-2'-O-Me-cAMP (8-pCPT) 를 처리하였을 때 HDAC6 의 발현이 증가하였다. 이소프로테레놀이나 8-pCPT을 처리하였을 때 EPAC의 하위인 Rap1의 활성이 증가하였다. 항시 활성형

Rap1A에 의해 HDAC6의 발현이 증가하였고, Rap1A의 발현을 저해하면, 이소프로테레놀의 의해 증가된 HDAC6의 발현이 감소하는 것을 확인하였다. H1299 세포에서 이소프로테레놀은 External signal-activated kinase (ERK) 를 억제하였다. ERK의 억제는 HDAC6의 발현을 증가시켰고, 항시 활성형 MAPK kinase (MEK1)에 의해서는 이소프로테레놀에 의해 증가된 HDAC6의 발현이 감소하였다. 따라서 이소프로테레놀은 PKA/Epac/ERK 신호 전달계를 통해 HDAC6의 발현을 증가시키고, 이소프로테레놀에 의해 유도된 HDAC6의 발현이 폐암세포의 이동성을 증가시킨다는 결론을 내렸다. 이 연구는 스트레스 신호가 HDAC6의 발현을 증가시켜 암세포의 이동성을 촉진할 수 있다는 것을 보여준다.

주요어: 스트레스 신호, Histone deacetylase 6 (HDAC6), Extracellular -regulated kinase 1/2, 세포 이동성

학번 : 2011-30621