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# Epinephrine Stimulates Esophageal Squamous-Cell Carcinoma Cell Proliferation Via β-Adrenoceptor-Dependent Transactivation of Extracellular Signal-Regulated Kinase/Cyclooxygenase-2 Pathway

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## ABSTRACT

Esophageal cancer is the sixth leading causes of cancer-related death in the world. It is suggested that β-adrenoceptor is involved in the control of cell proliferation, but its role in the pathogenesis of esophageal cancer remains unknown. We therefore studied the role of β-adrenergic signaling in the regulation of growth of an esophageal squamous-cell carcinoma cell line HKESC-1. Results showed that both $\beta_1$- and $\beta_2$-adrenoceptors were expressed in HKESC-1 cells. Stimulation of β-adrenoceptors with epinephrine significantly increased HKESC-1 cell proliferation accompanied by elevation of intracellular cyclic AMP levels, which were abolished by $\beta_1$- or $\beta_2$-selective antagonists. Epinephrine also increased extracellular signal-regulated kinase-1/2 (ERK1/2) phosphorylation as well as cyclooxygenase-2 (COX-2) and cytosolic phospholipase $A_2$ expression, which were blocked by $\beta_1$- or $\beta_2$-selective antagonists. Moreover, epinephrine increased cyclin $D_1$, cyclin $E_2$, cyclin-dependent kinase (CDK)-4, CDK-6, and $E_2F$-1 expression and retinoblastoma protein phosphorylation at Ser807/811, all of which were abrogated by $\beta_1$-adrenoceptor antagonist. Furthermore, epinephrine increased the expression of vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR)-1 and -2 in a $\beta_2$-adrenoceptor-, mitogen-activated protein kinase/ERK kinase (MEK)-, and COX-2-dependent manner. MEK or COX-2 inhibitor also significantly inhibited HKESC-1 cell proliferation induced by epinephrine. Collectively, we demonstrate that epinephrine stimulates esophageal squamous-cell carcinoma cell proliferation via β-adrenoceptor-dependent transactivation of ERK/COX-2 pathway. Stimulation of $\beta_1$- and $\beta_2$-adrenoceptors also elicits a differential response on the expression of cell cycle regulators. These novel findings may shed new light on the understanding of β-adrenergic signaling in the control of esophageal cancer cell growth. J. Cell. Biochem. 105: 53–60, 2008. © 2008 Wiley-Liss, Inc.

**KEY WORDS:** EPINEPHRINE; BETA-ADRENOCEPTOR; ESOPHAGEAL CANCER; CYCLOOXYGENASE; PROLIFERATION

Esophageal cancer represents one of the major morbidities and mortalities in the world. Currently, the global incidence is 2.5–5.0/100,000 for male and 1.5–2.5/100,000 for female [Pisani et al., 1999]. The incidence of esophageal cancer is even higher in areas such as China, Iran and Africa [Walker et al., 2002; Akbari et al., 2006; Su et al., 2007]. Based on histological classification, more than 90% of esophageal cancers can be categorized into squamous-cell carcinomas or adenocarcinomas. Both types of esophageal cancer pursue aggressive disease courses once the cancer developed, in which more than 50% of patients have either unresectable tumors or radiographically visible metastases at the time of diagnosis [Daly et al., 2000; Enzinger and Mayer, 2003]. The absence of early symptoms and rarity of hereditary pattern also render the population-based screening untenable in most part of the

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world. In a small subset of patients, surgery may provide cure but it is often preceded by high-dose debilitating chemotherapy [Goan et al., 2007]. The overall survival rate at 5 years is very poor and recent advances in the diagnosis, staging, and treatment of this neoplastic condition only have led to negligible improvements in survival [Daly et al., 2000; Enzinger and Mayer, 2003]. Esophageal cancer is the sixth leading cause of cancer-related death in the world [Pisani et al., 1999]. The cellular and molecular mechanism leading to the development of esophageal cancer, however, is not completely understood.

Our previous studies indicate that activation of β-adrenoceptors is involved in the regulation of proliferation of human gastric and colon cancer cells [Wu et al., 2005; Shin et al., 2007]. Other investigators also reported that activation of this G-protein-coupled receptor could modulate various cellular activities related to carcinogenesis. For instances, β-adrenergic stimulation has been shown to promote breast [Cakir et al., 2002], pulmonary [Schuller et al., 1999; Masi et al., 2005; Schuller and Cekanova, 2005] and pancreatic carcinoma [Weddle et al., 2001; Askari et al., 2005] cell growth as well as colon cancer cell migration [Masur et al., 2001]. Furthermore, propranolol, a β-adrenoceptor antagonist, reversed the anti-apoptotic effects of nicotinic stimulation on human lung cancer cells [Jin et al., 2004]. The involvement of β-adrenoceptors in carcinogenesis was further substantiated by the in vivo findings that β-adrenergic antagonists could retard the growth of melanoma and fibrosarcoma in nude mice xenograft model [Hasegawa and Saiki, 2002]. Moreover, a negative relationship between the use of β-blockers and cancer risk in human has been reported [Pahor et al., 1996; Algazi et al., 2004; Ronquist et al., 2004]. Although the importance of β-adrenergic signaling in the development of malignant diseases has been increasingly recognized, the exact cellular mechanism of β-adrenergic signaling in cancer is elusive. In this connection, emerging evidence suggested that arachidonic acid cascade might be involved in the promoting action of β-adrenoceptor activation [Schuller et al., 1999; Weddle et al., 2001; Wu et al., 2005].

Although β-adrenoceptor is thought to be involved in the control of cell proliferation, its expression and function in esophageal epithelial cells, however, remain unexplored. In the present study, we aim to delineate the role of β-adrenergic signaling in the regulation of the growth of a newly established esophageal squamous-cell carcinoma cell line HKESC-1. Through the use of differential β-adrenoceptor antagonists [both $\beta_1$- and $\beta_2$-], we try to elucidate the signaling pathways possibly mediated by β-adrenoceptors in relation to esophageal cancer growth.

## MATERIALS AND METHODS

### REAGENTS AND DRUGS

Epinephrine (an adrenoceptor agonist), atenolol (a specific $\beta_1$-adrenoceptor antagonist), ICI 118,551 (a specific $\beta_2$-adrenoceptor antagonist), PD98059 (a specific MEK inhibitor), Nimesulide (a highly selective cyclooxygenase-2 inhibitor), prostaglandin $E_2$ ($PGE_2$), and other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. Cytosolic phospholipase $A_2$ ($cPLA_2$), vascular endothelial growth factor (VEGF)-A 165, VEGF receptor-1 (VEGFR-1), VEGFR-2, cyclin-dependent kinases-4 (CDK-4), CDK-6, $E_2F$-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Extracellular signal-regulated kinases-1/2 (ERK1/2), phospho-ERK1/2 (Thr202/Tyr204), cyclin $D_1$, cyclin $E_2$, retinoblastoma (Rb), phospho-Rb (Ser807/811), phospho-Rb (Ser795), phospho-Rb (Ser780) antibodies were purchased from Cell Signaling Technology (Beverley, MA). Antibody specific to COX-2 was purchased from Cayman Chemical (Ann Arbor, MI).

### CELL CULTURE AND DRUG TREATMENT

HKESC-1 and HKESC-3 cell lines were established from a primary moderately-differentiated and a well-differentiated squamous cell carcinoma of the esophagus from a 47-year-old man and a 74-year-old Chinese man, respectively [Hu et al., 2000; Hu et al., 2002]. KYSE-150 cells, a human esophageal squamous cell carcinoma established from the poorly differentiated esophageal squamous cell carcinoma resected from a 49-year-old Japanese woman, were purchased from Japanese Collection of Research Bioresources Cell Bank (National Institute of Health Sciences, Tokyo, Japan). HKESC-1 and HKESC-3 cells were cultured in Minimum Essential Medium (Invitrogen, Carlsbad, CA) containing 10% and 20% fetal bovine serum (FBS) (Invitrogen), respectively, while KYSE-150 cells were cultured in F-12 (Ham) medium (Invitrogen) containing 10% FBS. Cells were cultured in the presence of 100 U/ml penicillin G, 100 μg/ml streptomycin, and maintained at 37°C, 95% humidity, and 5% carbon dioxide. Cells were plated at a density of $4 \times 10^4$ cells per well in 24-well plates. After 24 h of incubation for cell attachment, the cells were starved in 0.1% FBS-containing medium for another 12 h to synchronize the cell cycle. Epinephrine at different concentrations was incubated with the cells for 4 h to study the mitogenic effect of epinephrine. In order to examine the effects of various inhibitors, cells were pretreated with or without atenolol (100 μM), ICI 118,551 (50 μM), PD98059 (10 μM), Nimesulide (50 μM) for 45 min prior to epinephrine treatment.

### CELL PROLIFERATION ASSAY

Cell proliferation was measured as the amount of DNA synthesis using a modified [$^3$H]-thymidine incorporation assay. Cells were incubated in the absence or presence of epinephrine (10 μM) with or without different inhibitors for 4 h and then incubated with 0.5 μCi/ml [$^3$H]-thymidine (Amersham Corporation, Arlington Heights, IL) for another 4 h. The cells were then washed with ice-cold 0.15 M NaCl, followed by 10% trichloroacetic acid and incubated for 15 min at room temperature. After several washings, 1% SDS was added and incubated for another 15 min at 37°C. Finally, hydrophilic scintillation fluid was added into the vial and the amount of DNA synthesized was measured using liquid scintillation spectrometry on a beta-counter (Beckman Instruments, Fullerton, CA).

### CONVENTIONAL AND QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

The total cellular RNA was isolated from cells using Trizol reagent (Invitrogen). The RNA concentration was measured by GeneQuant II (Amersham Corporation) at 260 nm. Same amount of total RNA (5 μg) was used to generate the first strand of cDNA by reverse

transcription (Invitrogen) in accordance with the manufacturer's instructions. The polymerase chain reaction (PCR) primers were as follows: $\beta_1$-adrenoceptor, 5′-CTCCTTCTTCTGCGAGCTGT-3′ (sense), 5′-AGGATGGGCAGGAAGGAC-3′ (antisense); $\beta_2$-adrenoceptor, 5′-ACGCAGCAAAGGGACGAG-3′ (sense), 5′-CACACCATCAGAATGATCAC-3′ (antisense); The polymerase chain reaction (PCR) conditions were as follows: the template cDNA was first denatured at 94°C for 5 min. During 40 cycles of amplification, the denaturation step was at 94°C for 1 min, the annealing step was 56°C for $\beta_1$-adrenoceptor and 58°C for $\beta_2$-adrenoceptor for 1 min and the extension step at 72°C for 1 min. The final extension step was at 72°C for 7 min. The PCR products were electrophoresed on an agarose gel containing 0.5 μg/ml ethidium bromide.

For the determination of COX-2 and β-actin expression, quantitative PCR was performed with specific pre-designed primer set purchased from Qiagen. Conditions for quantitative PCR were 94°C for 5 min, 40 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min. Quantitative PCR was carried out using iQ SYBR Green Supermix (Bio-Rad) and Multicolor Real-Time PCR Detection System (Bio-Rad) as recommended by the manufacturer. The results were analyzed using the comparative threshold cycle ($C_T$) method, where $\Delta C_{T\ COX\text{-}2} = C_{T\ COX\text{-}2} - C_{T\ \beta\text{-actin}}$; and $\Delta\Delta C_{T\ COX\text{-}2} = \Delta C_{T\ COX\text{-}2\ (treated\ sample)} - \Delta C_{T\ COX\text{-}2\ (control\ sample)}$. The relative amount of COX-2-specific mRNA for each sample was calculated as $2^{-\Delta\Delta C_T}$. The specificity of PCR amplification was confirmed by DNA gel electrophoresis.

### WESTERN BLOT

Cells were harvested in radioimmunoprecipitation buffer [50 mmol/L Tris–HCl (pH 7.5), 150 mmol/L sodium chloride, 0.5% α-cholate acid, 0.1% SDS, 2 mmol/L EDTA, 1% Triton X-100, and 10% glycerol] containing proteinase and phosphatase inhibitors [1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM $Na_3VO_4$, and 1 mM NaF]. Protein was quantified using protein assay kit (Bio-Rad Laboratories, Hercules). Equal amounts of protein (40 μg/lane) were resolved by SDS–PAGE, and transferred to Hybond C nitrocellulose membranes (Amersham Corporation). The membranes were probed with primary antibodies overnight at 4°C and incubated for 1 h with secondary peroxidase-conjugated antibodies. They were developed with an enhanced chemiluminescence system (Amersham Corporation) and exposed to an X-ray film (FUJI Photo Film Co. Ltd., Tokyo, Japan). The correct molecular weight was confirmed with the molecular weight marker and quantitation was carried out with a video densitometer (Scan Maker III, Microtek).

### CYCLIC AMP ASSAY

Intracellular cyclic AMP (cAMP) assay was performed in accordance with the manufacturer's instructions. Briefly, $1 \times 10^6$ cells had been treated with or without epinephrine at 10 μmol/L for 30 min. The cAMP level was measured by EIA according to the manufacturer's recommendation (Amersham Pharmacia Biotech, Braunschweig, Germany). The cAMP level was expressed as picomoles per milligram of protein.

### STATISTICAL ANALYSIS

Results were expressed as the mean ± SEM with $n = 6$ for each sample for all paired statistical comparisons. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Turkey's *t*-test. *P* values less than 0.05 were considered statistically significant.

## RESULTS

### HKESC-1, HKESC-3, AND KYSE-150 CELLS EXPRESSED BOTH $\beta_1$- AND $\beta_2$-ADRENOCEPTORS

As stimulation of β-adrenoceptors has been shown to promote cancer cell proliferation [Weddle et al., 2001; Askari et al., 2005; Wu et al., 2005], the mRNA expression of $\beta_1$- and $\beta_2$-adrenoceptors was determined in HKESC-1, HKESC-3, and KYSE-150 esophageal squamous-cell carcinoma cell lines by reverse transcription-PCR. It was found that all three cell lines expressed the mRNAs of $\beta_1$- and $\beta_2$-adrenoceptors (Fig. 1). Thereafter, we chose HKESC-1 cells as the working cell line for further study in the subsequent experiments to examine the contributory role of β-adrenoceptors in cancer cell proliferation.

### EPINEPHRINE INCREASED HKESC-1 CELL PROLIFERATION WHICH WAS ABOLISHED BY β-ADRENOCEPTOR ANTAGONISTS

To study the effect of epinephrine on proliferation of esophageal squamous-cell carcinoma cells, we examined change in [$^3$H]-thymidine incorporation in response to epinephrine in cultured

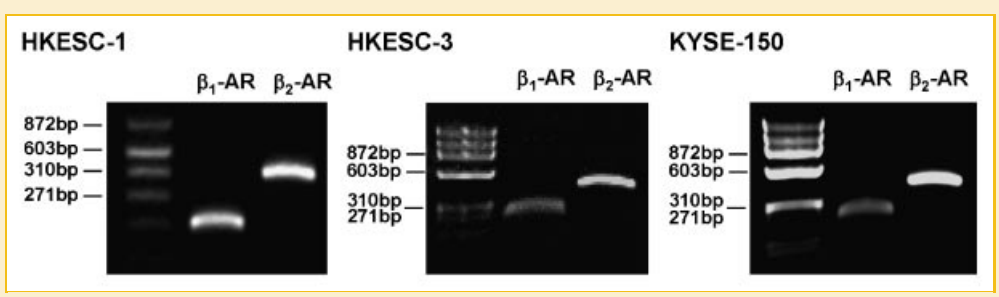


Fig. 1. Reverse transcription-PCR revealed the expression of $\beta_1$- and $\beta_2$-adrenoceptors mRNA in the esophageal squamous-cell carcinoma cell line HKESC-1, HKESC-3, and KYSE-150. The amplicon size of $\beta_1$- and $\beta_2$-adrenoceptor was 204 and 401 bp, respectively.

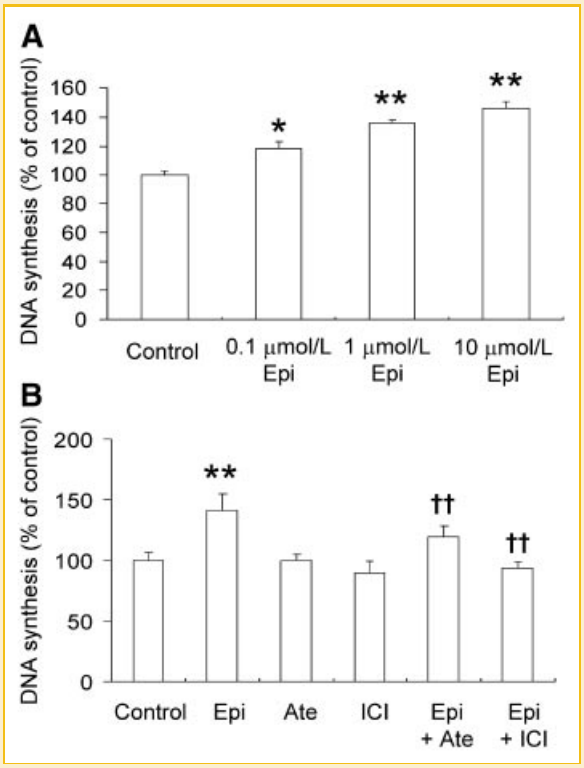


Fig. 2. A: The effect of 4-h treatment with epinephrine (Epi) on HKESC-1 DNA synthesis. Incubation with epinephrine dose-dependently increased DNA synthesis in HKESC-1 cells. B: The effect of $\beta_1$- and $\beta_2$-adrenoceptor blockade with atenolol (Ate, 100 μmol/L) and ICI 118,551 (ICI, 50 μmol/L) on epinephrine (Epi, 10 μmol/L)-induced HKESC-1 cell proliferation. HKESC-1 cells were pretreated with atenolol or ICI 118,551 for 45 min before incubation with epinephrine. Pretreatment with atenolol or ICI 118,551 reversed the epinephrine-induced cell proliferation. ICI 118,551 produced a more prominent effect in blocking the epinephrine-enhanced cell proliferation. $^{*}P < 0.05$; $^{**}P < 0.01$, significantly different from the untreated control group. $^{\dagger\dagger}P < 0.01$, significantly different from the epinephrine-treated group.

HKESC-1 cells. In Figure 2A, epinephrine enhanced [$^3$H]-thymidine incorporation into HKESC-1 cells in a dose-dependent manner in which the maximal stimulatory effect was observed at the dose of 10 μmol/L. This dose was thereafter applied in the subsequent experiments. To elucidate which receptor subtype mediates the stimulatory action, $\beta_1$-selective antagonist atenolol and $\beta_2$-selective antagonist ICI 118,551 were used. Both agents significantly dampened epinephrine-induced HKESC-1 cell proliferation (Fig. 2B), signifying the involvement of both $\beta_1$- and $\beta_2$-adrenoceptors in this stimulatory action. The cell viability in all treatment groups was confirmed to be comparable to that in the control group (>95% in all groups) by trypan blue exclusion assay.

## CYCLIC AMP LEVEL WAS ELEVATED UPON EPINEPHRINE TREATMENT IN HKESC-1

To determine whether activation of β-adrenoceptors in HKESC-1 cells could produce a functional response, intracellular levels of cAMP in HKESC-1 were measured. Ten micromolar of epinephrine produced a significant response on cAMP production in HKESC-1

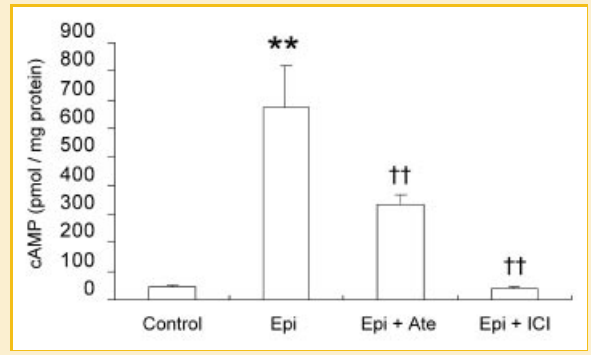


Fig. 3. The effects of 30-min treatment with epinephrine (Epi, 10 μmol/L) on HKESC-1 intracellular cAMP levels. Incubation with epinephrine significantly increased intracellular cAMP levels in HKESC-1 cells. Pretreatment with atenolol (Ate, 100 μmol/L) or ICI 118,551 (ICI, 50 μmol/L) reversed the epinephrine-induced elevation of intracellular cAMP levels. ICI 118,551 produced a more prominent effect than atenolol in blocking the epinephrine-stimulated intracellular cAMP levels. $^{**}P < 0.01$, significantly different from the untreated control group. $^{\dagger\dagger}P < 0.01$, significantly different from the epinephrine-treated group.

cells (Fig. 3). In this respect, epinephrine incubation at this concentration for 30 min markedly increased cAMP levels by about 15-fold. The increase in cAMP levels induced by epinephrine could be significantly reduced by $\beta_1$- or $\beta_2$-adrenoceptor antagonist. Nevertheless, the effect of $\beta_2$-adrenoceptor antagonist was more prominent than that of $\beta_1$-adrenoceptor antagonist.

## EPINEPHRINE INCREASED ERK12 PHOSPHORYLATION AS WELL AS $cPLA_2$ AND COX-2 EXPRESSION WHICH WERE ABOLISHED BY β-ADRENOCEPTOR ANTAGONISTS

Previous studies showed that phosphorylation of ERK1/2 and the subsequent activation of arachidonic acid cascade were involved in the stimulation of cell proliferation [Fan et al., 2001; Souza et al., 2004; Tominaga et al., 2004]. We therefore determined whether ERK1/2 phosphorylation as well as $cPLA_2$ and COX-2 expression in HKESC-1 cells, were increased upon epinephrine treatment. Results showed that epinephrine significantly increased ERK1/2 phosphorylation (Fig. 4A) as well as COX-2 and $cPLA_2$ protein expression in HKESC-1 cells, which were abolished by $\beta_1$- or $\beta_2$-adrenoceptor antagonist (Fig. 4B). Moreover, inhibition of MEK (a kinase acting directly upstream to ERK1/2), significantly abolished the stimulatory effect of epinephrine on ERK1/2 phosphorylation and COX-2 but not $cPLA_2$ protein expression. In parallel, the mRNA level of COX-2 was increased by about 70% in the epinephrine-treated HKESC-1 cells, which was also reversed by $\beta_1$-, $\beta_2$-adrenoceptor antagonist or MEK inhibitor (Fig. 4C). The protein expression of the total ERK1/2 was unaffected by any drug treatment in the current study (Fig. 4A).

## EPINEPHRINE INCREASED CYCLINS AND CDKs EXPRESSION AND Rb PHOSPHORYLATION WHICH WERE ABOLISHED BY $\beta_1$-ADRENOCEPTOR ANTAGONIST

Cell cycle progression requires orchestrated expression and interactions among different cell cycle regulators such as cyclins,

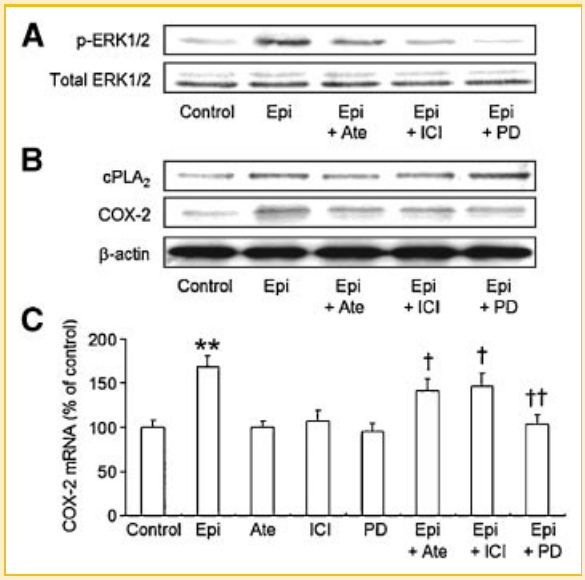


Fig. 4. A: Western blot analysis of ERK1/2 phosphorylation. Treating HKESC-1 cells with epinephrine (Epi, 10 μmol/L) for 15 min strongly increased ERK1/2 phosphorylation, which was abrogated by blockade of $\beta_1$- and $\beta_2$-adrenoceptors with atenolol (Ate, 100 μmol/L) and ICI 118,511 (ICI, 50 μmol/L), or inhibition of MEK with PD98059 (PD, 10 μmol/L), respectively. B: Western blot analysis of $cPLA_2$ and COX-2 protein expression. Treating the cells with epinephrine (Epi, 10 μmol/L) for 4 h increased the protein expression of $cPLA_2$ and COX-2, all of which were abolished by blockade of $\beta_1$- and $\beta_2$-adrenoceptors with atenolol (Ate, 100 μmol/L) and ICI 118,511 (ICI, 50 μmol/L) or inhibition of MEK with PD98059 (PD, 10 μmol/L), respectively. C: Expression of COX-2 mRNA in HKESC-1 cells was increased by epinephrine as revealed by real-time PCR analysis. Atenolol (Ate, 100 μmol/L), ICI 118,511 (ICI, 50 μmol/L), or PD98059 (PD, 10 μmol/L) reversed the effect. $^{**}P < 0.01$, significantly different from the control group. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$, significantly different from the epinephrine-treated group.

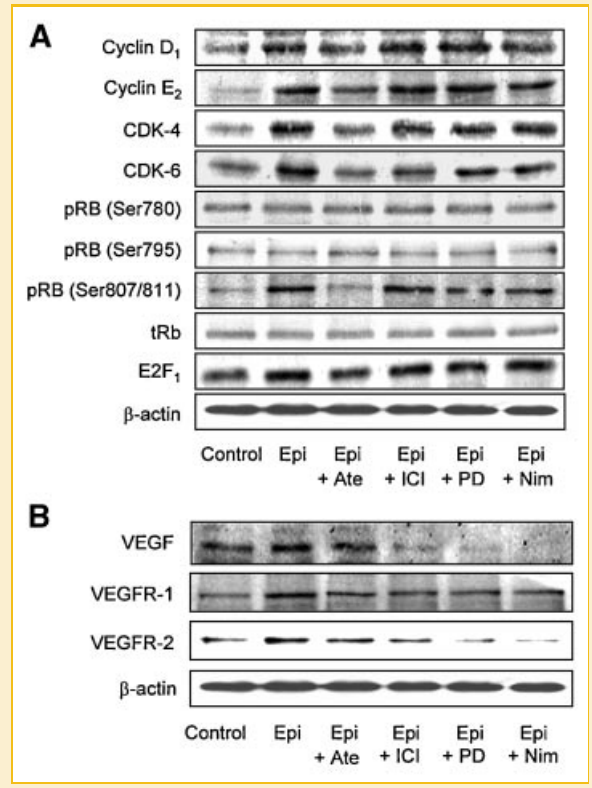


Fig. 5. A: Western blot analysis of cyclins and CDKs protein expression and Rb phosphorylation. Lane 1, control; lane 2, 10 μmol/L epinephrine (Epi); lane 3, 10 μmol/L epinephrine + 100 μmol/L atenolol (Ate); lane 4, 10 μmol/L epinephrine + 50 μmol/L ICI 118,551 (ICI); lane 5, 10 μmol/L epinephrine + 10 μmol/L PD98059 (PD); lane 6, 10 μmol/L epinephrine + 50 μmol/L Nimesulide (Nim). Epinephrine upregulated the protein expression of cyclin $D_1$, cyclin $E_2$, CDK-4, CDK-6, and $E_2F1$ as well as Rb phosphorylation at Ser807/811, which were reversed by blockade of $\beta_1$-adrenoceptor with atenolol. B: Western blot analysis of VEGF-A 165 and VEGFR protein expression. Lane 1, control; lane 2, 10 μmol/L epinephrine (Epi); lane 3, 10 μmol/L epinephrine + 100 μmol/L atenolol (Ate); lane 4, 10 μmol/L epinephrine + 50 μmol/L ICI 118,551 (ICI); lane 5, 10 μmol/L epinephrine + 10 μmol/L PD98059 (PD); lane 6, 10 μmol/L epinephrine + 50 μmol/L Nimesulide (Nim). Epinephrine upregulated the protein expression of VEGF-A 165, VEGFR-1 and -2, which were mainly abolished by blockade of $\beta_2$-adrenoceptor with ICI 118,551 or inhibition of MEK and COX-2 with PD98059 and Nimesulide, respectively.

CDKs, Rb, and $E_2F$-1. The effect of epinephrine on the expression of these cell cycle regulators is unknown. Results showed that epinephrine at the dose of 10 μmol/L significantly increased the expression of CDK-4, CDK-6, cyclin $D_1$, cyclin $E_2$, and $E_2F$-1. Moreover, epinephrine enhanced the phosphorylation of Rb at Ser 807/811, but not at Ser 780 or Ser 795 without affecting the total Rb expression Interestingly, $\beta_1$- but not $\beta_2$-adrenoceptor antagonist, MEK inhibitor, or COX-2 inhibitor abolished the expression of CDK-4, CDK-6, cyclin $D_1$, cyclin $E_2$, and $E_2F$-1 as well as Rb phosphorylation induced by epinephrine (Fig. 5A).

### EPINEPHRINE INCREASED VEGF AND VEGFR EXPRESSION WHICH WERE ABOLISHED BY $\beta_2$-ADRENOCEPTOR ANTAGONIST, ERK12 INHIBITOR, OR COX-2 INHIBITOR

VEGF is a key protein that modulates angiogenesis and tumor growth [Carmeliet and Jain, 2000]. Our results showed that 10 μmol/L epinephrine significantly increased VEGF, VEGFR-1 and -2 protein expression in HKESC-1 cells, which were abolished by $\beta_1$-, $\beta_2$-adrenoceptor antagonists, MEK inhibitor, or COX-2 inhibitor. However, the effect of $\beta_1$-antagonist was less prominent than those of other inhibitors (Fig. 5B).

### MEK AND COX-2 INHIBITORS ABOLISHED THE STIMULATORY EFFECT OF EPINEPHRINE ON CELL PROLIFERATION WHICH WAS REVERSED BY $PGE_2$

To confirm the involvement of ERK/COX-2 signaling in HKESC-1 cell proliferation induced by epinephrine, cells were treated with 10 μmol/L epinephrine in the absence or presence of MEK and COX-2 inhibitors with or without $PGE_2$ supplement. Results revealed that MEK or COX-2 inhibitors significantly abrogated the stimulatory effect of epinephrine on HKECS-1 cell proliferation, which were reversed by exogenous $PGE_2$. Interestingly, $PGE_2$ preferentially reversed the inhibitory effect of $\beta_2$- but not $\beta_1$-adrenoceptor antagonist (Fig. 6).

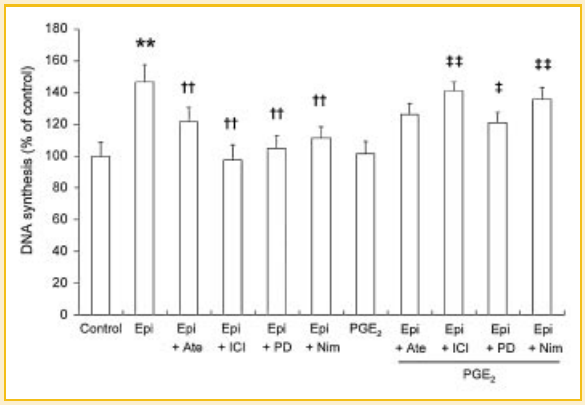


Fig. 6. Selective blockade of $\beta_1$- and $\beta_2$-adrenoceptors with atenolol (Ate, 100 μmol/L) and ICI 118,551 (ICI, 50 μmol/L) or inhibition of MEK and COX-2 with PD98059 (PD, 10 μmol/L) and Nimesulide (Nim, 50 μmol/L) respectively abolished HKESC-1 cell proliferation induced by epinephrine (Epi, 10 μmol/L). Exogenous PGE2 (1 nmol/L) significantly reversed the abolishing effects of ICI 118,551, PD98059, and Nimesulide. $^{**}P < 0.01$, significantly different from the untreated control group. $^{††}P < 0.01$, significantly different from the epinephrine-treated group. $^{‡}P < 0.05$; $^{‡‡}P < 0.01$, significantly different from respective epinephrine + inhibitor-treated group.

## DISCUSSION

Stimulation of β-adrenoceptors has been implicated in the promotion of cancer cell growth [Weddle et al., 2001; Askari et al., 2005; Wu et al., 2005], but its involvement in the proliferation of esophageal cancer was undetermined. In the present study, we demonstrate that $\beta_1$- and $\beta_2$-adrenoceptors were expressed in esophageal squamous-cell carcinoma cell lines (Fig. 1). Stimulation of β-adrenoceptor with epinephrine significantly increased intracellular cAMP concentration (Fig. 3) and promoted cell proliferation (Fig. 2), which could be reversed by $\beta_1$- or $\beta_2$-adrenoceptors. All these evidence suggest that stimulation of β-adrenoceptors promotes cell proliferation via a functional signal transduction pathway. Moreover, blockade of $\beta_2$- but not $\beta_1$-adrenoceptor completely abolished epinephrine-induced cell proliferation, indicating that the mitogenic effect of epinephrine was predominately mediated through $\beta_2$-adrenoceptor. This finding is consistent with our previous report that the stimulatory effect of a cigarette smoke component 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone on colon cancer cell proliferation was preferentially mediated by the $\beta_2$ subtype of adrenoceptors [Wu et al., 2005]. Moreover, although the regulatory function of β-adrenoceptors on cell proliferation has been reported in cancers of diverse origins, including cancers of the lungs, pancreas, breast, stomach, and colon [Schuller et al., 2000; Cakir et al., 2002; Askari et al., 2005; Schuller and Cekanova, 2005; Wu et al., 2005; Shin et al., 2007], all of these malignancies are adenocarcinomas. Here we provide the first evidence that β-adrenergic stimulation also promotes the cell proliferation of squamous-cell carcinoma.

β-Adrenergic stimulation has been reported to promote cancer growth via activation of arachidonic cascade [Weddle et al., 2001; Wu et al., 2005]. The exact mechanism bridging β-adrenoceptor activation and arachidonic cascade is unknown. In this study, we demonstrate for the first that epinephrine stimulates cell proliferation of esophageal squamous-cell carcinoma via β-adrenoceptor-dependent transactivation of ERK/COX-2 signaling pathway where blockade or inhibition of either component in the pathway significantly reversed the stimulatory effect of epinephrine. In this respect, our results showed that the phosphorylation of ERK1/2 induced by epinephrine was abolished by β-adrenoceptor antagonists (Fig. 4), suggesting that ERK1/2 phosphorylation was under the control of β-adrenoceptor in HKESC-1 cells. Moreover, β-adrenoceptor antagonists or MEK inhibitor significantly reduced epinephrine-induced COX-2 expression (Fig. 4), indicating that COX-2 was downstream to β-adrenoceptor and ERK1/2. The mechanism by which β-adrenoceptor activation leads to ERK1/2 phosphorylation and COX-2 induction is unknown. However, it has been proposed that β-adrenoceptor activation could increase the kinase activity of c-Src whose activation is known to induce Ras/Raf/MEK/ERK signaling [Fredriksson et al., 2000; Karni et al., 2005]. On the other hand, cAMP-dependent activation of protein kinase A has been shown to induce ERK1/2 phosphorylation via Rap1/B-raf/MEK pathway [Peyssonnaux and Eychène, 2001; Dumaz and Marais, 2005; Wang et al., 2006]. To this end, phosphorylation of ERK1/2 has been shown to increase COX-2 expression via induction of early growth response genes c-Fos and c-Jun [Guo et al., 2001; Shin et al., 2007]. Another interesting finding of the present study was that $\beta_1$- or $\beta_2$-adrenoceptor antagonist but not MEK inhibitor altered the expression of $cPLA_2$ (Fig. 4), indicating that the signaling pathways regulating COX-2 and $cPLA_2$ expression diverged at a point between β-adrenoceptors and MEK. Interestingly, we also notice that the expression of $cPLA_2$ is slightly higher in epinephrine-stimulated cells when MEK inhibitor is present. The molecular mechanism underlying this observation, however, is unknown but it is worthwhile to notice that arachidonic acid release depends not only on the expression but also the activity of $cPLA_2$, which is governed by phosphorylation. In relation to cAMP production, it is observed that $\beta_2$-adrenoceptor antagonist is sufficient to reduce the intracellular cAMP concentration to the level of control, rendering the role of $\beta_1$-adrenoceptor in this process more elusive. In this respect, it has been reported that $\beta_1$- and $\beta_2$-adrenoceptor can interact with each other to form heterodimer [Zhu et al., 2005], posing the possibility that $\beta_2$-adrenoceptor antagonist may interfere with the coupling of $\beta_1$-adrenoceptor to adenylyl cyclase in the event of agonist binding.

Cell cycle progression requires a coordinated expression of and interactions among different cyclins and CDKs [Wikman and Kettunen, 2006]. For instances, cyclin $D_1$-CDK-4/6 and cyclin $E_2$/CDK-2 complexes phosphorylate Rb protein at different serine and threonine residues, leading to the release of $E_2F$ transcription factors that initiate the transcription of genes which are necessary for $G_1$–S transition [Wikman and Kettunen, 2006]. β-adrenergic stimulation has been shown to increase cell proliferation in various cell line studies but its effects on the expression of different cell cycle regulators in cancer have not yet been described [Weddle et al., 2001; Askari et al., 2005; Wu et al., 2005]. In this study, we showed that epinephrine treatment resulted in a moderate to strong increase

in the protein levels of CDK-4, CDK-6, cyclin $D_1$, and cyclin $E_2$ in esophageal cancer cells. A similar trend was also observed for the phosphorylation levels of Rb at Ser807/811, but not at Ser780 and Ser795, and the protein expression level of $E_2F$-1 (Fig. 5A). More noteworthy was that all of the cell cycle regulators induced by epinephrine were mainly reversed by $\beta_1$-adrenoceptor antagonist, but not by $\beta_2$-adrenoceptor antagonist or COX-2 inhibitor, which seemed to have more potential than $\beta_1$-adrenoceptor antagonist to abolish the mitogenic effect of epinephrine on HKESC-1 cells. This novel observation implicates that different but overlapped signaling pathways might be engaged by $\beta_1$- and $\beta_2$-adrenoceptors. The mechanism by which selective $\beta_1$-adrenoceptor activation induced the expression of these cell cycle regulators, however, was presently unknown. Moreover, although $\beta_2$-adrenergic stimulation had no effect on the expression of these factors, it is possible that activation of $\beta_2$-adrenoceptors could modify their interactions or regulate other cell cycle-related factors which are more important in epinephrine-induced esophageal cancer cell proliferation. In this connection, we also notice that MEK or COX-2 inhibitors did not reverse the upregulation of cell cycle regulators induced by epinephrine. This finding suggests that the effect of epinephrine on the expression of cell cycle regulators is MEK- and COX-2-independent.

Epidemiologic and experimental animal studies have shown that psychological stress may promote tumor growth [Reynolds and Kaplan, 1990; Ben-Eliyahu et al., 1999; Kojima et al., 2005; Antoni et al., 2006; Thaker et al., 2006]. However, the biological significance of stress and its pathogenic mechanisms in human malignant diseases are still a matter of controversy. Epinephrine has been shown to be elevated in individuals with acute or chronic stress but the effect of this stress hormone on cancer growth has not yet been well characterized [Schmidt and Kraft, 1996]. In the present study, results revealed that epinephrine directly stimulated esophageal cancer cell proliferation via β-adrenoceptor/ERK/COX-2 pathway and $\beta_1$-adrenoceptor-dependent upregulation of cyclins and CDKs. Interestingly, results also showed that the catecholamine epinephrine could increase VEGF expression in esophageal cancer cells via a $\beta_2$-adrenoceptor-, ERK1/2- and COX-2-dependent pathway (Fig. 5B), suggesting that epinephrine might further enhance the growth of the tumor through the promotion of angiogenesis. This finding is in line with the results of an in vivo study that chronic stress increases angiogenesis and tumor growth of ovarian cancer through the $\beta_2$-adrenergic activation of the cAMP/protein kinase A signaling pathway and the subsequent upregulation of VEGF, matrix metalloproteinases-2, and -9 [Thaker et al., 2006]. Moreover, our results showed that the expression levels of VEGFR-1 and -2 were increased by epinephrine in a $\beta_2$-adrenoceptor-, ERK1/2- and COX-2-dependent manner. The pathophysiological significance of this observation is still unclear. Nevertheless, it is possible that the upregulation of VEGFR may render the cancer cells more sensitive to the autocrine stimulation of VEGF. With respect to this, overexpression of VEGFR has been evidenced in many types of cancer and stimulation of these receptors on cancer cells is known to modulate cancer cell growth and metastasis [Dales et al., 2004; Shin et al., 2005; Hara et al., 2006; Nakayama et al., 2006; Yamaguchi et al., 2007].

To date, β-adrenoceptors have been reported to take part in the promotion and progression of different kinds of neoplasm [Weddle et al., 2001; Askari et al., 2005; Wu et al., 2005; Shin et al., 2007]. We here also present a unique picture of β-adrenoceptor-mediated signaling in relation to esophageal squamous cancer cell proliferation. Our experimental data along with the findings of other investigators [Weddle et al., 2001; Askari et al., 2005; Wu et al., 2005; Shin et al., 2007] not only shed new light on the understanding of the carcinogenic mechanism in catecholamine-related cancer, but may also open up a new chemoprophylactic and therapeutic possibility of the use of β-adrenoceptor antagonists for the prevention and treatment of esophageal cancer particularly related to squamous-cell carcinomas.

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