Inducible cAMP Early Repressor Inhibits Growth of Vascular Smooth Muscle Cell

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Objective—The role of inducible cAMP early repressor (ICER), a transcriptional repressor, in the vascular remodeling process has not been determined. We examined whether ICER affects growth of vascular smooth muscle cells (VSMCs).

Methods and Results—Semi-quantitative RT-PCR and Western blot analysis showed that expression of ICER was increased in beraprost (a prostaglandin I₂ analogue)-stimulated VSMCs in a time- and dose-dependent manner. The induction of ICER was inhibited by pretreatment with H89, a protein kinase A (PKA) inhibitor, suggesting that PKA mediates the induction of ICER expression. Beraprost suppressed platelet-derived growth factor–induced thymidine incorporation in VSMCs, which was reversed by transfection of short interfering RNA for ICER, not by scramble RNA.

Overexpression of ICER by an adenovirus vector attenuated neointimal formation (intima/media ratio) by 50% compared with overexpression of LacZ. The number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling–positive cells was increased and the number of Ki-67–positive cells was decreased in ICER-transduced artery.

Conclusion—These results suggest that ICER induces apoptosis and inhibits proliferation of VSMCs, and plays a critical role in beraprost-mediated suppression of VSMC proliferation. ICER may be an important endogenous inhibitor of vascular proliferation. (Arterioscler Thromb Vasc Biol. 2007;27:1549-1555.)

Key Words: PGI₂ analogue ■ inducible cAMP early repressor ■ neointimal formation ■ VSMC

Transcription of many cellular genes is regulated by changes of intracellular cAMP levels. The increased cAMP activates protein kinase A (PKA), resulting in the PKA-dependent phosphorylation of nuclear proteins that belong to the cAMP response element (CRE)-binding protein (CREB) family of transcription factors, such as CREB, cAMP response element modulator (CREM), and activating transcription factor-1. Inducible cAMP early repressor (ICER), an isoform of CREM, has a DNA binding domain but lacks a transactivation domain. Therefore, ICER binds to CRE sites but inhibits CRE-dependent gene transcription, which makes ICER serve as a transcriptional repressor. Expression of ICER is inducible in various cell types such as pituitary cells, and cardiac myocytes.

Activation of CREB family protein is generally mediated by phosphorylation. Activity of ICER, however, depends on the level of its expression, because ICER dose not contain a PKA-dependent phosphorylation site. Although Yehia et al previously reported that ICER was phosphorylated by mitogen activated protein kinase (MAPK), phosphorylation of ICER affected stability of ICER protein without an effect on ICER activity.

Beraprost, a prostaglandin I₂ (PGI₂) analogue, has anti-platelet and vasodilatory effects. Beraprost is, therefore, clinically used for the treatment of arteriosclerosis obliterans and pulmonary hypertension. Beraprost functions through cell surface G protein–coupled PGI₂ receptor designated IP receptor. Activation of IP receptor increases intracellular cAMP level via stimulation of adenylyl cyclase. Activation of adenylyl cyclase increases intracellular cAMP level, which is followed by PKA activation.

It has been reported that beraprost suppresses platelet-derived growth factor (PDGF)-induced DNA synthesis of vascular smooth muscle cells (VSMCs). However, a key regulatory molecule of beraprost-mediated suppression of PDGF-stimulated DNA synthesis has not been determined. The role of ICER in blood vessels and atherogenesis is also poorly characterized. We showed in the present study that beraprost suppresses PDGF-stimulated DNA synthesis through induction of ICER, and overexpression of ICER suppresses neointimal formation in balloon-injured rat carotid artery.

Materials and Methods

Materials

Dulbecco modified Eagle medium (DMEM) was purchased from Gibco BRL, and fetal bovine serum (FBS) were purchased from JRH
Biosciences Inc. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse IgG) were purchased from VECTOR Laboratories Inc. A rabbit polyclonal antibody against CREM-1 for detection of ICER was obtained from Santa Cruz Biotechnology. An anti-CREB antibody was purchased from Cell Signaling Technology. An antibody against Ki-67 antigen was provided by Astellas Pharma Inc. Other chemical reagents were purchased from Wako Pure Chemicals unless specifically mentioned.

Cell Culture
VSMCs were isolated from the thoracic aorta of Sprague-Dawley rat by an explant method and maintained in DMEM supplemented with 10% FBS in a humidified atmosphere of 95% air-5% CO₂ at 37°C. VSMCs were cultured until grown to confluence, cultured in DMEM with 0.1% BSA for additional 2 days, and used in the experiment. Beraprost sodium was provided by Astellas Pharma Inc. Other chemical reagents were purchased from Wako Pure Chemicals unless specifically mentioned.

Adenovirus Vector Expressing ICER and LacZ
A recombinant adenovirus vector expressing ICER (AdICER) was reported previously. Confluent VSMCs were washed 2 times with PBS, cultured in DMEM with 0.1% BSA for 2 days, and used for the experiments. Multiplicity of infection (moi) indicates the number of virus per cell added to a culture dish.

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction
Total RNA was prepared according to an acid guanidinium-phenol-chloroform extraction method. Total RNA was phenol-chloroform-extracted and ethanol-precipitated. Then, the total RNA (0.4 µg) was reverse-transcribed (RT) using molony murine leukemia virus reverse transcriptase (ReverTra Ace-α kit, TOYOBO) in 4 µL of reaction mixtures. Semi-quantitative PCR was performed with a T3000 Thermocycler (Biometra) according to the manufacturer’s instruction. An aliquot of RT-reaction mixture (0.5 µL for amplification of ICER and 0.2 µL for amplification of Glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) was subjected to PCR. The sequences for sense and antisense primers for ICER were 5'-CTT TAT TTT GGA CTG TGT TAC GG-3' and 5'-TAC TAA TCT GTT TTG GGA GAG CA-3', respectively. GAPDH was used as a reference for the amount of cDNA. The sequences for sense and antisense primers for GAPDH were 5'-TTC TTG TGC AGT GCC AGC CTC GTC-3' and 5'-TAG GAA CAG GGA AGG CGG CGA CCC TGC CAG-3', respectively. Appropriate cycles for ICER and GAPDH were determined to confirm the linear amplification of cDNA by PCR (data not shown). Thirty cycles for ICER and 24 cycles for GAPDH were used. The cDNAs of ICER and GAPDH after PCR reaction were electrophoresed on 2% agarose gel, and stained with ethidiumbromide. The density of ICER and GAPDH cDNA visualized on 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore). The blots were blocked with TBS-T (20mmol/L Tris-HCl [pH 7.6], 10% glycerol, 2% SDS, 0.05% bromophenolblue, and 715mmol/L 2-mercaptoethanol) at 95°C for 3 minutes, electrophoresed on 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore). The blots were blocked with TBS-T (20mmol/L Tris-HCl [pH 7.6], 137mmol/L NaCl, 0.1% Tween 20) containing 5% skim milk at room temperature for 30 minutes. Western blot analysis of ICER and α-tubulin were performed as described previously.

Short Interfering RNA
The annealed form of siRNA of ICER was constructed from a 19 to 21 bp of ICER (NCBI nucleotide accession number S66024) by
Samchully Pharm Co. The optimal ICER sequence was 5′-CUU AUA GAG GAG CUU GAA A-3′. A scrambled sequence, 5′-GAG UAC UUA AGG AUG ACU ATT-3′, that does not contain any significant homology to ICER sequence was used as a control. Introduction of siRNA and scrambled RNA (scrRNA) for ICER to VSMCs was performed by lipofection method (Lipofectamine 2000, Invitrogen Co) according to the manufacturer’s instruction.

**Measurement of CRE-Dependent Gene Promoter Activity**

VSMCs (5×10^5) were prepared in a 6-cm tissue culture dish. Five μg of CRE (3 copies)-luciferase fusion DNA with thymidine kinase gene promoter and 2 μg of β-galactosidase gene were introduced to VSMCs by lipofection method according to the manufacturer’s instruction (Invitrogen Co) with siRNA or scRNA for ICER. Measurement of CRE-dependent gene promoter activity was performed as described previously.15

**Measurement of DNA Synthesis**

siRNA for ICER was introduced into VSMCs by lipofection method. After introduction of siRNA, VSMCs were washed with PBS, and cultured in DMEM with 0.1% BSA for 2 days. VSMCs were pretreated with or without beraprost (1 μmol/L) for 1 hour. VSMCs were then stimulated with or without PDGF-BB (50 ng/mL) for 24 hours. The cells were labeled with [3H]-thymidine (PerkinElmer Life Sciences) for the last 4 hours. After labeling, the cells were washed with PBS, fixed in 10% trichloroacetic acid (TCA), and then washed with a mixture of ethanol and ether (2:1). The cells were lysed in 0.5N NaOH, and incorporated [3H]-thymidine was measured by a scintillation counter.

**Detection of Apoptosis In Vitro**

VSMCs were stimulated with beraprost (1 μmol/L), or infected with AdLacZ (100 moi) or AdICER (100 moi) and maintained in DMEM supplemented with 0.1% BSA for 2 days. VSMCs were isolated through trypsinization. The isolated cells and cells in the medium were collected by centrifugation and stained with propidium iodide (PI). The number of hypodiploid cells was counted from 10,000 cells with Fluorescence-activated cell sorting (FACS; EPICS ALTRA MulticOMP, Beckman Coulter) analysis as described previously.16

**Balloon Injury Model and Infection With Adenovirus**

All procedures were approved by the institutional animal use and care committee, and were conducted in conformity with institutional guidelines. Balloon injury and infection with adenovirus were performed as described previously.16 Male Sprague-Dawley rats (Kyudo Co, Japan) (370 to 400g) were anesthetized by intraperitoneal administration of pentobarbital sodium. The left common artery was denuded of the endothelium with 2F Fogarty balloon catheter (Baxter) that was introduced through the external carotid artery. Inflation and retraction of the balloon catheter were repeated 3 times. AdICER (5×10^9 plaque forming unit [PFU]) or AdLacZ (5×10^9PFU) was introduced into the lumen, and the carotid artery was incubated for 15 minutes without blood flow. Then, the viral solution was removed, and the blood flow was restored.

**Morphometry and Detection of Apoptosis and Cell Proliferation In Vivo**

Morphometry was performed as described previously.16 Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method with an apoptosis in situ detection kit (Wako Pure Chemicals). The counterstain was hematoxylin. Cell proliferation was examined by immunohistochemistry with anti–Ki-67 antibody (Dako Inc), which was a nuclear protein preferentially expressed during all active phase of the cell cycle (G1, S, G2, and M phases), but absent in resting (G0) cells.17

In brief, after the rats were killed, the carotid artery was excised, and fixed with methacarn (methanol: chloroform: acetic acid=6:3:1). After fixation, the carotid artery was embedded into paraffin. The samples were sectioned serially at 4 μm thickness, fixed in acetone, and stained immunohistochemically with an anti–Ki-67 antibody.
Quantitative analysis was performed from 100 cells in 5 independent sections from each rat (n = 6).

**β-Galactosidase Staining In Vivo**

The balloon-injured rat carotid artery were stained with the β-galactosidase staining buffer, which contains 5mmol/L K₄Fe(CN)₆, 5mmol/L K₃Fe(CN)₆, and 1% 5-bromo-4-chloro-3-indole-β-D-galactosidase (X-gal) in PBS, for 6 hours at 37°C. These arteries are fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS, and embedded into paraffin. The samples were serially sectioned at 4 μm thickness and fixed in acetone. Hematoxylin and eosin were used as counterstaining.

**Statistical Analysis**

Statistical analysis was performed with 1-way or 2-way ANOVA and Fisher test if appropriate. P<0.05 was considered to be statistically significant. Data are shown as mean±SEM.

**Results**

**Beraprost Induces Expression of ICER mRNA**

To examine whether ICER is induced in VSMCs, VSMCs were incubated with beraprost (1μmol/L) for various periods, and expression of ICER mRNA was determined by semi-quantitative RT-PCR method. Two species of ICER mRNA (I and II), which are produced by alternative splicing, were detected. Both bands were taken into account for the densitometric analysis. Maximum expression of ICER mRNA was observed at 3 hours after stimulation (Figure 1A, 1B).

Expression of ICER mRNA was dose-dependently increased by beraprost (Figure 1C, 1D).

**Beraprost Induces Expression of ICER Protein**

VSMCs were incubated with beraprost (1μmol/L) for various periods, and expression of ICER protein was determined by Western blot analysis. Maximum expression of ICER protein was observed at 3 hours after stimulation (Figure 2A, 2B). Expression of ICER protein was dose-dependently increased by beraprost (Figure 2C, 2D). Two bands represented ICER/I γ or ICER II/ICER II γ, which were produced by alternative splicing of γ exon.18
Beraprost Induces Expression of ICER mRNA Through PKA Pathway

We examined whether PKA pathway mediates induction of ICER mRNA by beraprost. VSMCs were stimulated with beraprost for 3 hours after pretreatment with or without H89, a PKA inhibitor. H89 significantly inhibited the expression of ICER mRNA induced by beraprost (Figure 3A, 3B). To examine whether beraprost-induced expression of ICER mRNA requires de novo protein synthesis, we examined the effect of cycloheximide (CHX). Pretreatment with CHX (10 μg/mL) did not affect induction of ICER mRNA by beraprost (Figure 3C, 3D), suggesting that de novo protein synthesis is not required. We examined whether beraprost affects ICER mRNA stability. Beraprost did not affect the degradation rate of ICER mRNA (Figure 3E), suggesting that beraprost does not affect ICER mRNA stability.

ICER Mediates Beraprost-Induced Suppression of VSMC Proliferation

Previously, it was reported that beraprost inhibited VSMC growth. We examined the role of ICER in beraprost-induced growth suppression. To knockdown of ICER expression, we used siRNA technique. Introduction of siRNA for ICER sufficiently inhibited the expression of beraprost-induced ICER protein, compared with that of scRNA (supplemental Figure IA and IB, available online at http://atvb.ahajournals.org). Neither ICER siRNA nor scRNA affected CREB expression. Introduction of siRNA for ICER significantly upregulated beraprost-induced CRE-luciferase activity (supplemental Figure IC), suggesting that ICER negatively regulates CRE-dependent gene transcription induced by beraprost. Beraprost attenuated [3H]-thymidine incorporation into VSMCs induced by PDGF, and overexpression of ICER also inhibited PDGF-induced VSMCs proliferation. ICER siRNA prevented the inhibitory effect of beraprost (supplemental Figure ID), suggesting that ICER is a key regulatory molecule in the inhibitory effect of beraprost on VSMC proliferation. In addition, overexpression of ICER induced apoptosis of VSMCs. However, beraprost did not increase the number of apoptotic cells (supplemental Figure IE). This may be attributable to that ICER inhibits its expression through negative feedback mechanism, and therefore ICER expression induced by beraprost is transient (Figure 1).

Overexpression of ICER Attenuates Neointimal Formation in Balloon-Injured Artery

A previous report showed that beraprost inhibited neointimal formation after balloon injury. We examined whether overexpression of ICER showed the same effect. We overexpressed ICER in balloon-injured artery by infection with AdICER. Balloon injury decreased endogenous ICER expression (Figure 4A and 4B), although the difference was statis-
Discussion

In the present study, we showed that ICER is inducible in VSMCs by beraprost. We also examined whether ICER is involved in beraprost-induced growth suppression of VSMCs in vitro. The critical role of ICER in beraprost-induced growth suppression was clarified by the experiment using siRNA for ICER, which showed that downregulation of beraprost-induced ICER expression abolished the growth inhibition by beraprost. Overexpression of ICER suppressed neointimal formation in balloon-injured rat carotid artery through induction of apoptosis and inhibition of proliferation.

It was reported that beraprost inhibits neointimal formation by preventing the downregulation of p27Kip1 expression, a cyclin-dependent kinase inhibitor that is downregulated by denudation in a canine coronary artery injury model. In this study, beraprost also reduced the number of cells in S phase and increased the number of cells in G1 phase in EGF-stimulated cultured VSMCs, indicating that the cell cycle arrest in G1 phase was induced by beraprost. Lames et al reported that overexpression of ICER induced G2/M arrest in pituitary corticotroph cell line through direct downregulation of the cyclin A, which contains functional CRE sites in the promoter region. In addition, there are several other mechanisms by which cAMP cascades inhibit VSMC growth. Cospedal et al previously reported that cAMP elevating agents inhibited activation of extracellular signal-regulated protein kinases (ERK) 1/2 induced by PDGF. Another group reported that accumulation of cAMP inhibited PDGF-stimulated VSMC growth through apoptosis by induction of p21 and anti-oncogene p53. These results suggest that beraprost may inhibit PDGF-induced proliferation of VSMCs through induction of cell cycle arrest at these 2 cell cycle check points and apoptosis. Because CREB is known to regulate the expression of proliferating cellular nuclear antigen, cyclin A, and cyclin D1, it may be possible that ICER inhibits these cell cycle check points.

We previously reported that CREB mediated tumor necrosis factor (TNF)-α–induced migration of VSMCs. Therefore, it may be possible that overexpression of ICER inhibits VSMC migration from media to neointima, resulting in the attenuation of neointimal formation. Intriguingly, the promoter region of TNF-α that is produced from macrophages as well as VSMCs contains functional CRE site, and another group reported that TNF-α induction through CRE-binding site in cardiac fibroblasts, suggesting that TNF-α production from injured artery may be suppressed by ICER overexpression.

Tomita et al reported that overexpression of ICER in cardiac myocytes induced apoptosis through downregulation of Bcl-2, an antiapoptotic molecule. We also reported that overexpression of dominant negative CREB induced VSMCs apoptosis through Bcl-2 downregulation. These data suggest that overexpression of ICER in the injured artery may suppress the Bcl-2 expression in the neointimal VSMCs, and result in an increase in the number of apoptotic cells. Adhesion of mononuclear cells to dysfunctional endothelium is considered to be an early step of the atherosclerosis. Various adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, E-selectin, and intercellular adhesion molecule are shown to be expressed in atherosclerotic lesion and are involved in this cell attachment. Beraprost is reported to inhibit TNF-α–induced expression of VCAM-1 in cultured endothelial cells. Because overexpression of dominant negative CREB also inhibits TNF-α–induced VCAM-1 expression, beraprost may inhibit VCAM-1 expression through induction of ICER.

In the present study, we showed that overexpression of ICER inhibited neointimal formation through apoptosis and growth inhibition of VSMCs in vivo. The promoter region of ICER contains 4 CRE-like elements known as cAMP-autoregulatory element. The induction of ICER expression is mediated by CREB, and the induced ICER inhibits ICER induction through CRE site. Thus, this negative feedback system limits the expression of ICER. It is possible that the self-limited induction may weaken the antigrowth effect of endogenous ICER. Although the mechanism is not clear, balloon injury attenuated the expression of ICER, which may contribute to neointimal formation. Therefore the forced expression of ICER in injured artery may be more effective in preventing neointimal formation.

In conclusion, we showed in the present study that ICER suppresses proliferation of VSMCs and induces apoptosis. Our data suggest that ICER may be a novel therapeutic tool for vascular proliferative diseases.

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Disclosures
None.

References


