

Adenovirus Vector E4 Gene Regulates Connexin 40 and 43 Expression in Endothelial Cells via PKA and PI3K Signal Pathways

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Abstract—Connexins (Cxs) provide a means for intercellular communication and play important roles in the pathophysiology of vascular cardiac diseases. Infection of endothelial cells (ECs) with first-generation E1/E3-deleted E4⁺ adenovirus (AdE4⁺) selectively modulates the survival and angiogenic potential of ECs by as of yet unrecognized mechanisms. We show here that AdE4⁺ vectors potentiate Cx expression in ECs in vitro and in mouse heart tissue. Infection of ECs with AdE4⁺, but not AdE4⁻, resulted in a time- and dose-dependent induction of junctional Cx40 expression and suppression of Cx43 protein and mRNA expression. Treatment of ECs with PKA inhibitor H89 or PI3K inhibitor LY294002 prevented the AdE4⁺-mediated regulation of Cx40 and Cx43 that was associated with diminished AdE4⁺-mediated survival of ECs. Moreover, both PKA activity and cAMP-response element (CRE)-binding activity were enhanced by treatment of ECs with AdE4⁺. However, there is no causal evidence of a cross-talk between the 2 modulatory pathways, PKA and PI3K. Remarkably, Cx40 immunostaining was markedly increased and Cx43 was decreased in the heart tissue of mice treated with intra-tracheal AdE4⁺. Taken together, these results suggest that AdE4⁺ may play an important role in the regulation of Cx expression in ECs, and that these effects are mediated by both the PKA/CREB and PI3K signaling pathways. (*Circ Res.* 2005;96:950-957.)

Key Words: endothelial cells ■ connexin ■ PI3K ■ PKA ■ adenovirus

E⁴⁺ adenoviral (Ad) vector infection of endothelial cells (ECs) results in activation and increased survival.^{1,2} These AdE4⁺-mediated changes in vascular ECs may contribute to the vascular toxicities observed during treatment with adenoviral vectors. We have previously reported that E4⁺ genes induce engagement of the junctional adhesion molecule vascular endothelial-cadherin, which results in activation of PI3K/Akt pathway and thereby supporting the survival of the ECs.² However, our additional data obtained from microarray analysis demonstrated that other junctional proteins, connexins, are also substantially modulated during AdE4⁺ infection, suggesting that alteration in signaling through these junctional proteins may also play a role in AdE4⁺-mediated survival of ECs.

Connexins (Cxs) are a group of at least 20 highly conserved proteins that provide the basis for communication through the direct exchange of ions, nutrients, second messengers, electrical coupling, and small metabolites from one cell to neighboring cells.^{3,4} This intercellular communication plays an important role in controlling cell homeostasis, transmigration, proliferation, differentiation,

and apoptosis.^{5,6} Recent animal experimental and clinical evidence increasingly shows Cxs contribute to the regulation of blood flow, vasomotor activity, microvessel development, and functional maintenance of vasculature.⁷ Cxs are particularly associated with development in vascular tissue and are functionally relevant in vascular and heart diseases.⁸ Vascular endothelium expresses at least 3 connexin isoforms, namely connexin 37 (Cx37), connexin 40 (Cx40), and connexin 43 (Cx43).^{7,9,10} Cx37 and Cx40 knockout mice display severe vascular abnormalities and cardiac malformations.¹¹⁻¹³ Reducing Cx43 expression in Cx43^{+/-} mice impedes progression of atherosclerosis by inhibition of leukocyte infiltration into the lesions,¹⁴ and decreased expression of Cx43 has also been reported as a common feature that is associated with increased arrhythmias and heart diseases.¹⁵⁻¹⁷ Moreover, loss of Cx40 facilitates arterial hypertension.¹² It is therefore important to identify factors that modulate the expression of these particular Cxs in endothelial cells.

In this study, we show that AdE4⁺, but not AdE4⁻ vectors, modulate the expression various Cxs. Remark-

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ably, AdE4⁺ vectors induce Cx40 expression but suppress Cx43 expression in primary ECs and mouse heart tissue. These data suggest that in addition to the activation of junctional adhesion molecule VE-cadherin, induction of Cx40 expression, and suppression of Cx43 expression in endothelial cells through the PKA and PI3K signaling pathways support AdE4⁺-mediated EC survival.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described¹⁸ and cultured in EC medium [M199 medium containing 10% (v/v) fetal bovine serum, 20 μ g/mL EC growth factor, 20 U/mL heparin, 100 μ g/mL penicillin, and 100 μ g/mL streptomycin] in a humidified incubator at 37°C with air/5% CO₂. HUVEC monolayers, passages 2 to 4, were used in these studies. Cell viability was assayed by the trypan blue exclusion method and indicated that fewer than 5% of the cells absorbed the dye both before and after infection with Ad vectors.

Construction of Ad Vectors

The following Ad vectors were constructed: AdE4⁺: AdLacZ(E4⁺) (E1⁻, E2⁺, E3⁻, E4⁺, CMV promoter driving the *Escherichia coli* β -galactosidase [β -gal] gene)¹⁹ or AdNull(E4⁺) (E1⁻, E2⁺, E3⁻, E4⁺, CMV promoter driving no transgene in the expression cassette); and AdE4⁻: AdLacZ(E4⁻) (same as AdLacZ, but with a complete deletion of the E4 gene, using the β -gal gene as a spacer in the E4 region).

Western Blot Analysis

Cells were lysed in RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 10 μ g/mL aprotinin). Insoluble debris was pelleted, and the protein concentration of the supernatant was determined using a DC protein assay kit (Bio-Rad). Fifty micrograms of each protein sample were separated on 10% SDS-PAGE gels. The protein samples were then transferred to nitrocellulose membrane. Protein expression was determined by immunoblotting with the following antibodies: anti-Cx40 (CHEMICON International), anti-Cx43 (Sigma), and anti- β -actin (Sigma). After incubation with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies, the membranes were developed with enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology).

Northern Blot Analysis

Total RNA from HUVECs was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions.²⁰ Total RNA (10 μ g) was separated on 1.0% agarose gels containing 2.2 mol/L formaldehyde, transferred to nylon membranes, and UV cross-linked. Probes for cDNA of Cx40 or Cx43 were radiolabeled with [γ -³²P]dCTP using a random primer DNA labeling kit. Northern hybridization was done using QuickHyb (Stratagene) as per the manufacturer's protocol. After hybridization, the membranes were washed twice in 0.2% SSC containing 0.1% (wt/vol) SDS at room temperature for 20 minutes and finally in 0.1% SSC/0.1% SDS at 42°C for 45 minutes. The membranes were exposed to Kodak film.

PKA Activity Assay

PKA activity in cellular lysates was assayed using a PKA assay kit according to the manufacturer's instructions (Upstate Biotechnology).²¹ HUVECs were lysed in Buffer A as described by the kit manufacturer and 200 μ g of lysate protein were incubated for 30 minutes at 30°C with 100 μ mol/L Kemptide, a PKA-specific substrate, and 10 μ Ci of [γ -³²P]ATP in kinase buffer provided by the kit manufacturer. The phosphorylated substrate was separated from the residual [γ -³²P]ATP using P81 phosphocellulose paper and quantitated using a liquid scintillation counter.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed using a DNA-protein binding detection kit (Promega) according to the manufacturer's protocol. Briefly, the nuclear extracts of transfected cells were extracted with the NE-PER nuclear extraction reagent (Pierce). Double-stranded oligonucleotides for cAMP-responsive element-binding protein (CREB) (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3') were labeled with [γ -³²P]ATP by T4 polynucleotide kinase (Promega). The extracts were then analyzed by nondenaturing 4% polyacrylamide gel electrophoresis in 0.5 \times TBE buffer at 200 V for 30 minutes. The gel was dried and exposed to X-ray film.

Immunofluorescence

HUVECs were cultured on gelatin-coated plastic coverslips and treated with AdE4⁺ or PBS for 2 days. Cells were fixed in 1% freshly prepared paraformaldehyde for 10 minutes at room temperature and permeabilized by adding (final concentrations) 0.1% Triton X-100 and 0.1% bovine serum albumin for 5 minutes at room temperature. The fixed cells or paraffin embedded tissues from mouse heart were incubated overnight at 4°C in 10% goat serum and then with primary antibodies for Cx40 or Cx43 at 1:100 dilution for 1 hour at room temperature. The cells were then washed 3 times with PBS and then incubated with rhodamine-conjugated goat anti-mouse antibody at 1:200 dilution for 1 hour at room temperature. Coverslips were washed with PBS and counterstained with DAPI (1 μ g/mL) to visualize the nuclei. Fluorescence was visualized with an Olympus BX51 microscope.

In Vivo Experiments

C57Bl/6 mice, 8 to 10 weeks old, weighing 18 to 20 g, were used for this study (Charles River, Wilmington, Mass; and Taconic,

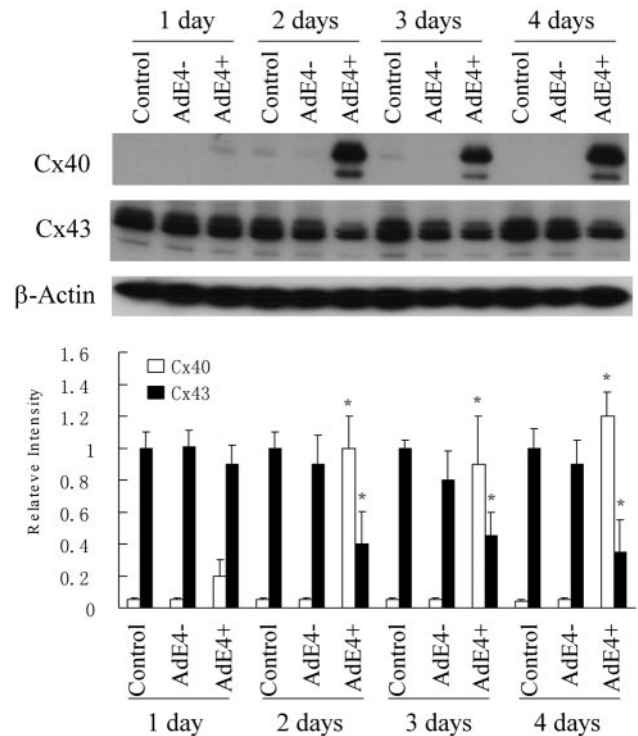


Figure 1. AdE4⁺ alter Cx40 and Cx43 protein expression in ECs. ECs were treated with PBS (control), AdE4⁻ 10⁴ particles/cell, and AdE4⁺ 10⁴ particles/cell, and cultured in growth factor- and serum-free medium for the indicated time points. Cellular extracts were prepared from ECs, the cell lysates were then analyzed by immunoblot using polyclonal anti-connexin 40 (Cx40), polyclonal anti-connexin 43 (Cx43), and polyclonal anti- β -actin. Bottom, Densitometric analysis of the Cx40 and Cx43. Solid bar, Cx40; open bars, Cx43. Results represent mean \pm SE of 3 separate experiments. **P* < 0.01 compared with control.

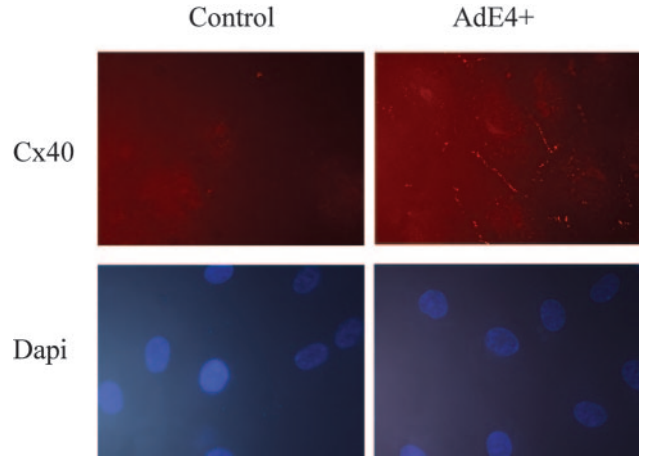
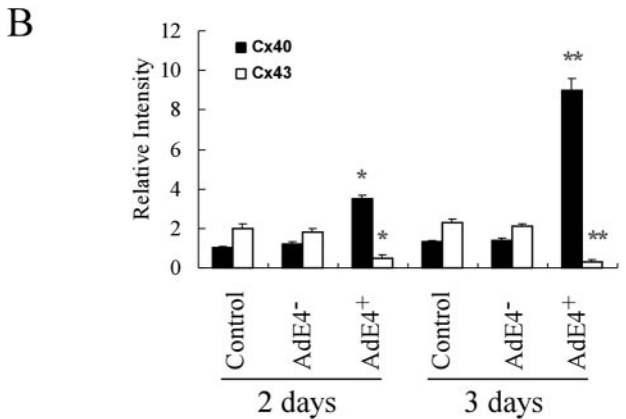
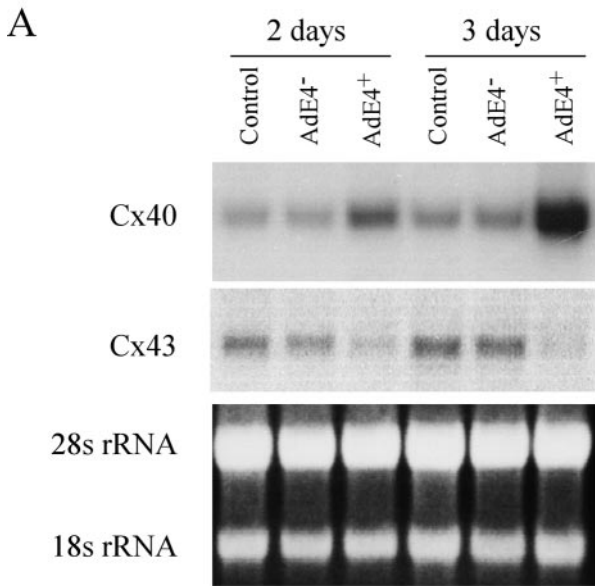


Figure 3. Effect of AdE4⁺ on Cx40 localization in endothelial cells. ECs were exposed to PBS (control) or AdE4⁺ for 10⁴ particles/cell for 2 days. Cells were fixed and probed with anti-Cx40 antibody followed by fluorescein-conjugated secondary antibody; nuclei are stained with DAPI. Image was visualized using fluorescence microscopy. Results are representative of 3 separate experiments.

5- μ m thickness were cut and placed on glass microscope slides for immunostaining.

Results

AdE4⁺ Regulates Cx40 and Cx43 Protein and mRNA Expression in ECs

To determine whether AdE4⁺ modulates Cx expression in ECs, HUVECs were infected with AdE4⁺ or AdE4⁻ vectors and the expression of Cxs was determined by Western Blot analysis. As shown in Figure 1, infection with AdE4⁺ but not AdE4⁻ vectors increased Cx40 protein expression in a time-dependent manner, with maximal induction of Cx40 expression observed between 2 and 4 days. Upregulation of Cx40 was dose-dependent with maximal effect observed between 1 \times 10⁴ and 2 \times 10⁴ particles/cell of AdE4⁺ used (data not shown). In contrast to Cx40, Cx43 is abundantly expressed in HUVECs under normal conditions. Remarkably, infection with AdE4⁺ significantly suppressed Cx43 protein expression in HUVECs at 2 to 4 days (Figure 1).

In order to investigate whether AdE4⁺ regulates Cxs at the transcriptional level, we performed Northern blot analysis for Cx40 and Cx43 on AdE4⁻ and AdE4⁺-treated HUVECs. As shown in Figure 2, Cx40 mRNA was induced 2 to 3 days after infection with AdE4⁺ but not with AdE4⁻. Conversely, Cx43 mRNA was significantly decreased after infection with AdE4⁺, but not with AdE4⁻, at 2 to 3 days of EC infection.

Effect of AdE4⁺ on Cellular Distribution of Cxs in ECs

To examine the effects of AdE4⁺ on cellular trafficking of Cx40 and Cx43, HUVECs were treated with AdE4⁺ or control (PBS) for 2 days before Cx40 and Cx43 localization was detected by immunofluorescence. No significant staining for Cx40 was observed in untreated HUVECs, whereas infection with AdE4⁺ resulted in markedly in-

Figure 2. Effect of AdE4⁺ on Cx40 and Cx43 mRNA expression in ECs. A, Total RNA (10 μ g/lane) was isolated from uninfected control cells and cells infected with AdE4⁻ (10⁴ particles/cell) or AdE4⁺ (10⁴ particles/cell) for 2 to 3 days. Blot was hybridized to a ³²P-labeled human Cx40 and Cx43 cDNA probe. Equal RNA loading was demonstrated by staining with ethidium bromide-stained 18S and 28S rRNA. B, Northern bands of Cx40 and Cx43 intensities were scanned, and relative Cx40 and Cx43 mRNA levels were determined by normalization to the 18s rRNA signal quantified by densitometry. Filled bar, Cx40; open bars, Cx43. Results represent mean \pm SE of 3 separate experiments. **P*<0.05 compared with control, ***P*<0.01 compared with control.

Germantown, NY). Animals were anesthetized with 250 to 300 μ L of a 1:10 dilution of ketamine (Fort Dodge Animal Health) and xylazine (Bayer) by intraperitoneal injection. After exposing the trachea, a 25-gauge angiocath was inserted under direct vision through the tracheal wall into the lumen of the midportion of the trachea. AdE4⁺ was resuspended in phosphate-buffered saline (PBS), pH 7.4, and diluted to a final volume of 50 μ L, which was slowly injected through the angiocath over a period of 1 to 2 minutes. To assess expression of Cxs in the heart, the mouse hearts were extracted 5 days after AdE4⁺ administration. The mice were deeply anesthetized, and hearts were excised and fixed in 4% paraformaldehyde (PFA; 0.1 mol/L phosphate buffer, pH 7.4) for 3 hours. Subsequently, the hearts were transferred into 70% ethanol overnight and then embedded in paraffin. The heart tissue was paraffin-embedded and butterfly-shaped sections of

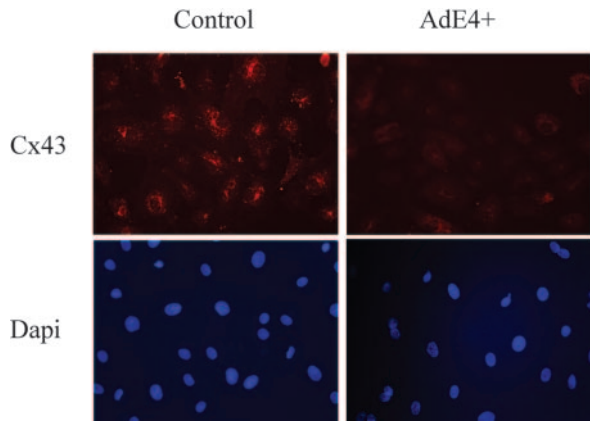


Figure 4. Effect of AdE4⁺ on Cx43 localization in endothelial cells. HUVECs were exposed to PBS (control) or AdE4⁺ for 10⁴ particles/cell (B) for 2 days. Cells were fixed and probed with anti-Cx43 antibody followed by fluorescein-conjugated secondary antibody. Nuclei are stained with DAPI. Image was visualized using fluorescence microscopy. Results are representative of 3 separate experiments.

creased staining of Cx40 on the cell surface of ECs (Figure 3). In contrast, robust Cx43 expression was observed in uninfected control cells. Infection with AdE4⁺ resulted in significant downregulation of Cx43 staining (Figure 4). These data validate the results of Western and Northern blot studies, which demonstrate that AdE4⁺ vectors induce Cx40 expression while downregulating Cx43 localization to the membrane.

Role of PKA and CREB in AdE4⁺-Regulated Cx Expression

To determine whether AdE4⁺ infection of ECs mediates Cx expression via activation of PKA and cAMP pathways, HUVECs were treated with the PKA inhibitor H89, adenylylate cyclase activator forskolin. As shown in Figure 5A and 5B, H89 significantly blocked AdE4⁺-mediated Cx40 induction and Cx43 suppression. However, forskolin did not significantly alter the effect of AdE4⁺ on Cx40 or Cx43 expression under the time and dose conditions. To analyze whether AdE4⁺ could induce activation of PKA, we assessed the effects of AdE4⁺ on PKA activity. As shown in Figure 5C, AdE4⁺ treatment in ECs induced a significant increase in PKA activity, whereas pretreatment with inhibitors of PKA, H89 or KT5720, inhibited AdE4⁺-induced activation of PKA. Because PKA phosphorylates and activates the transcription factor CREB, which binds to a consensus sequence that regulates many CREB-targeted genes, we investigated whether CREB was capable of binding such a consensus sequence after infection of ECs with AdE4⁺. The nuclear extracts from AdE4⁺-treated ECs were analyzed by EMSA. As shown in Figure 5D, AdE4⁺ increased CREB DNA binding in ECs. These data suggest that PKA/CREB-dependent signaling may play a role in AdE4⁺-regulated Cx expression.

Role of the PI3K/Akt Signaling Pathway in AdE4⁺-Regulated Cx Expression

We have previously demonstrated that one mechanism by which AdE4⁺ vectors support survival of ECs is through

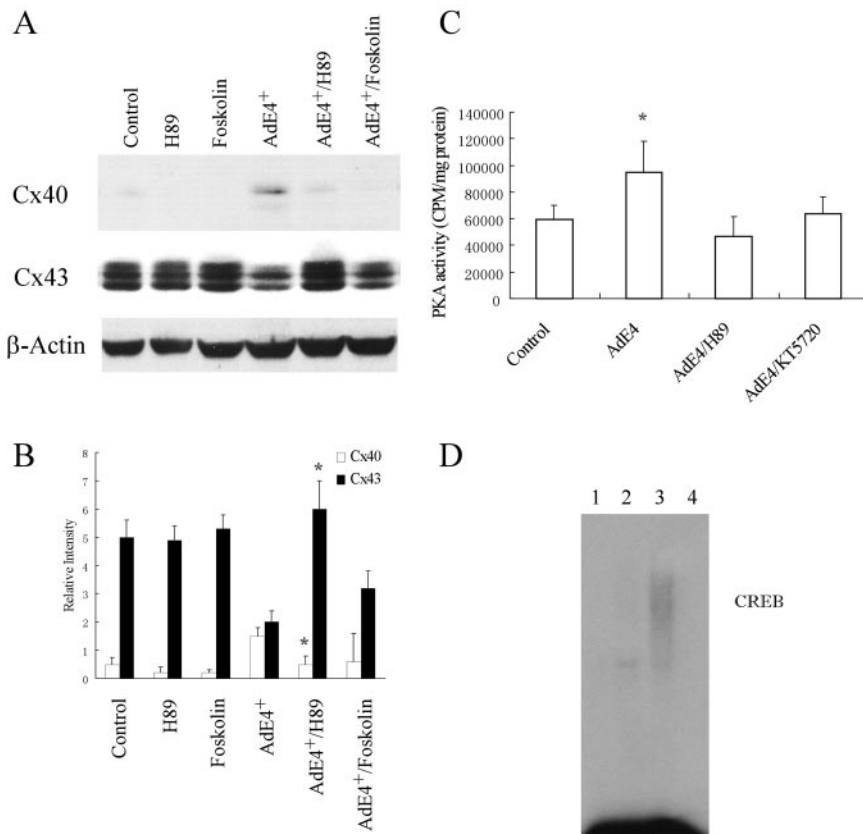


Figure 5. AdE4⁺ regulates of Cx40 and Cx43 expression in ECs by increasing PKA activity and CREB DNA binding activity. A, HUVECs were infected with AdE4⁺ (10⁴ particles/cell) or uninfected control and cotreated with either H89 (5 μmol/L) or forskolin (10 μmol/L) for 48 hours. Cells were harvested and lysed for Western blot analysis for Cx40, Cx43, and β-actin expression. B, Densitometric analyses of Western blots of Cx40 and Cx43 are presented as mean ± SE for 3 independent experiments performed in triplicate, *P < 0.05 compared with AdE4⁺. C, AdE4⁺ enhances PKA activity in HUVECs. HUVECs were infected with AdE4⁺ (10⁴ particles/cell) or uninfected control and cotreated with either H89 (5 μmol/L) or KT5720 (5 μmol/L) for 48 hours. PKA activity, measured as incorporation of [γ-³²P]ATP, was determined for each sample in the presence or absence of Kemp-tide substrate. Results represent mean ± SE of 3 separate experiments; *P < 0.05 compared with control. D, Effect of AdE4⁺ on CREB DNA binding in HUVECs. Nuclear protein (2 μg) was incubated with the radiolabeled oligonucleotide containing CREB consensus sequence for analysis by EMSA. Lane 1, AdE4⁺ treatment, 24 hours, sample treated with 100× unlabeled CREB oligonucleotide; Lane 2, PBS control treatment, 24 hours; Lane 3, AdE4⁺ treatment, 24 hours; Lane 4, free probe alone (no nuclear extracts).

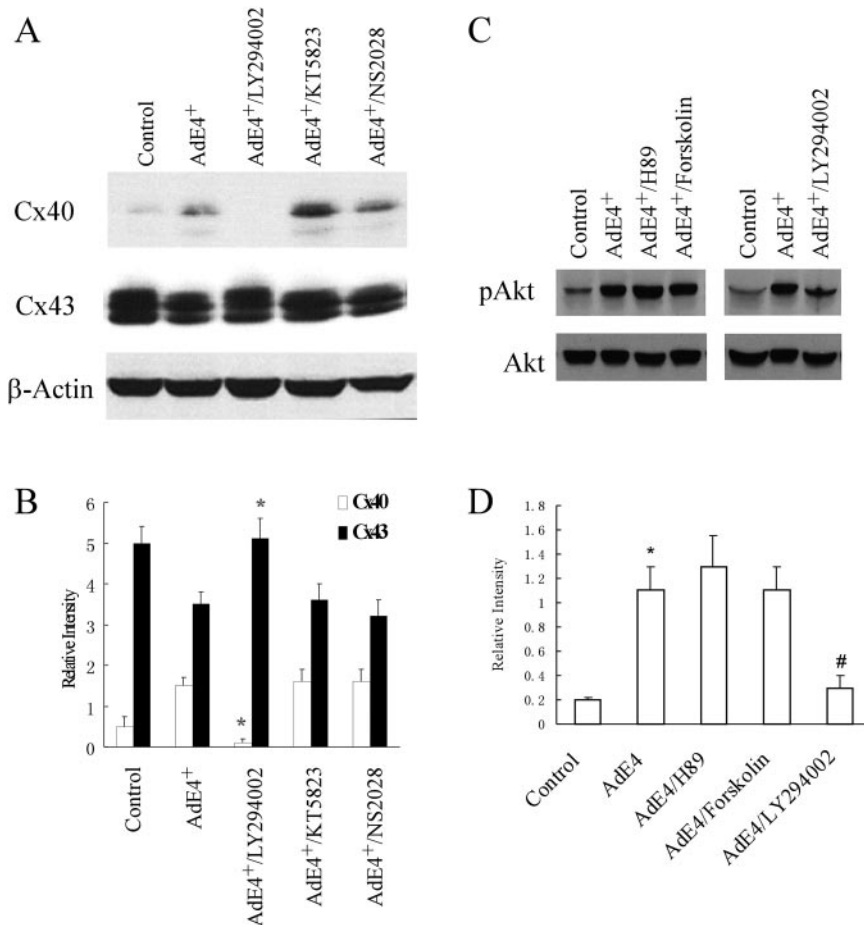


Figure 6. Effect of kinase inhibitors and forskolin on AdE4⁺-mediated Cx40, Cx43, and pAkt expression in endothelial cells. A, HUVECs were infected with AdE4⁺ (10⁴ particles/cell) or uninfected control and cotreated with either LY294002 (10 μmol/L), KT 5823 (1 μmol/L), and NS 2028 (1 μmol/L) for 48 hours. Cells were harvested and lysed for Western blot analysis for Cx40, Cx43, and β-actin expression. B, Densitometric analyses of Western blot of Cx40 and Cx43 are presented as mean±SE for 3 independent experiments performed in triplicate; **P*<0.05 compared with AdE4⁺. C, HUVECs were infected with AdE4⁺ (10⁴ particles/cell) or uninfected control and cotreated with either H89 (10 μmol/L), forskolin (1 μmol/L), or LY 294002 (10 μmol/L) for 48 hours. Cells were harvested and lysed for Western blot analysis for pAkt and total Akt expression. D, Densitometric analyses of Western blot of pAkt and Akt are presented as mean±SE for 3 independent experiments performed in triplicate; **P*<0.05 compared with control; #*P*<0.05 compared with AdE4⁺.

activation of the PI3K/Akt pathway.² We thus investigated whether this signal cascade was involved in the regulation of Cx expression in AdE4⁺-infected ECs. Figure 6A and 6B show that treatment of ECs with PI3K inhibitor LY294002 (10 μmol/L) abrogated both the induction of Cx40 and suppression of Cx43 by AdE4⁺, whereas soluble guanylyl cyclase inhibitor NS2028 and specific PKG inhibitor KT5823 did not significantly alter the effect of AdE4⁺ on Cx40 or Cx43 expression (Figure 6A and 6B), suggesting that the effect of AdE4⁺ on Cx expression depends on activation of the PI3K/Akt signaling cascade.

To determine whether AdE4⁺ modulation of Cxs may involve possible cross-talk between PI3K and PKA signaling pathways, Akt phosphorylation was detected after EC treatment with forskolin or H89. As shown in Figure 6C and 6D, neither forskolin nor H89 pretreatment of ECs infected with AdE4⁺-affected phosphorylation of Akt, whereas LY294002 faithfully inhibited Akt phosphorylation in ECs. This indicates that activation of the PI3K by AdE4⁺ is not directly dependent on PKA signaling.

Degradation of Cx43 in ECs Does Not Require Proteasome

Cx43 is characterized by a moderately rapid half-life of ≈1 to 5 hours.^{22,23} We tested whether the decrease in Cx43 protein expression by AdE4⁺ is associated with Cx43 protein degradation via the ubiquitin-proteasome pathway.

ECs were infected with AdE4⁺ in the presence of a protein synthesis inhibitor cycloheximide (CHX) and proteasome inhibitors lactacystin or MG132. As shown in Figure 7A, CHX inhibited 90% of the Cx43 protein expression with infection of AdE4⁺. However, neither lactacystin nor MG132 (data not shown) protected against decreased levels of Cx43 protein by AdE4⁺ in either the presence or absence of CHX, suggesting that proteasome activity is not required for AdE4⁺-induced Cx43 in heart tissue downregulation.

Pertussis Toxin–Sensitive G Proteins Mediate Cx Expression

G-coupled proteins mediate signal transduction conveyed through activation of receptors localized in the cell membrane by activating soluble second messenger molecules or by altering plasma membrane channel function. To assess whether G_{iα} and G_{sα} are involved in regulation of Cxs, HUVEC monolayers were pretreated with an inhibitor of G_{iα} pertussis toxin (PTX, 100 ng/mL) or an inhibitor of G_{sα} cholera toxin (CTX, 1 μg/mL), then treated with AdE4⁺ or control for 2 days. As seen in Figure 7B, treatment with PTX, but not CTX, significantly attenuated baseline expression level of Cx40 and Cx43 in control cells. Pretreatment with either PTX or CTX abolished the AdE4⁺-induced Cx40 expression. However, pretreatment with PTX and CTX had no significant impact on AdE4⁺-

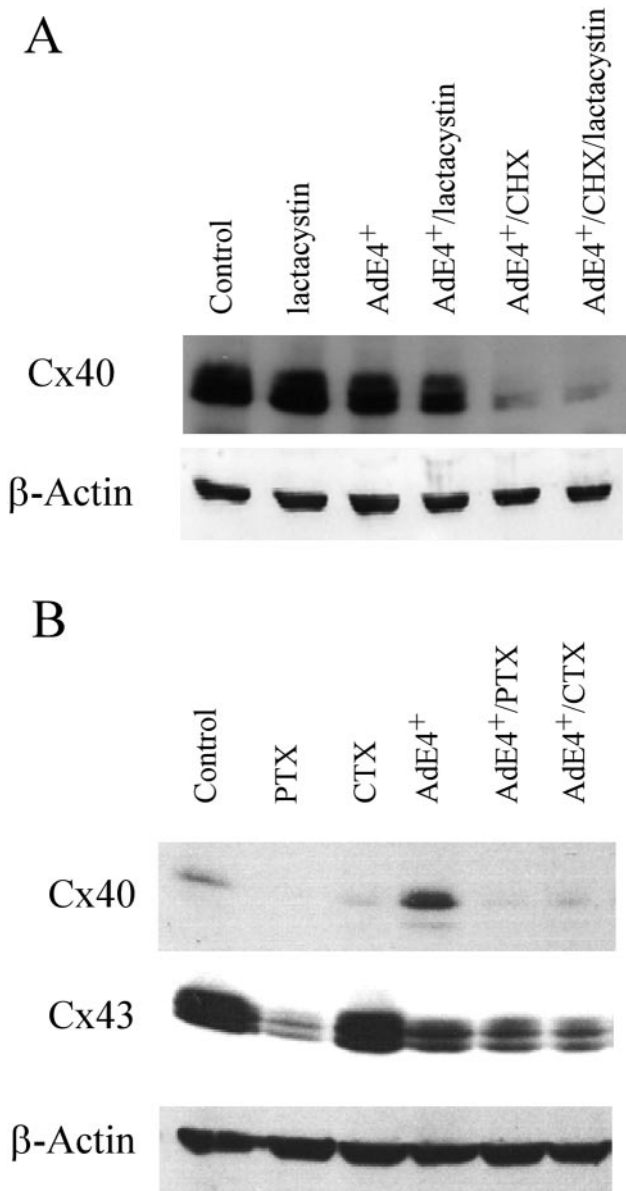


Figure 7. Effect of lactacystin, cycloheximide, pertussis toxin, or cholera toxin on Cx40 and Cx43 expression in endothelial cells. A, HUVECs were infected with AdE4⁺ (10^4 particles/cell) or uninfected control and cotreated with either lactacystin ($10 \mu\text{mol/L}$) and cycloheximide (CHX) ($1 \mu\text{g/mL}$). After 24 hours, cells were harvested and lysed for Western blot analysis of Cx43 and β -actin expression. B, HUVECs were infected with AdE4⁺ (10^4 particles/cell) or uninfected control and cotreated with either pertussis toxin (PTX) (100 ng/mL) and cholera toxin (CTX) ($1 \mu\text{g/mL}$). After 48 hours, cells were harvested and lysed for Western blot analysis of Cx40, Cx43, and β -actin expression.

mediated Cx43 downregulation. These results indicate that PTX-sensitive and CTX-sensitive G proteins may have different signaling effects on Cx40 and Cx43. Cx40 may require both $G_{i\alpha}$ and $G_{s\alpha}$ subunits, whereas Cx43 may be dependent on only the activation of PTX-sensitive $G_{i\alpha}$.

Induction of Cx40 and Suppression of Cx43 by AdE4⁺ in Mouse Heart Tissue

Cxs exist abundantly in cardiac vascular tissue and play important roles in myocardial function. Therefore, we

examined whether AdE4⁺ affects Cx40 and Cx43 protein distribution in mouse heart tissue. Five days after intratracheal administration of AdE4⁺ to the mouse lungs, the expression levels of Cx40 and Cx43 in heart tissue were determined by Western blot and immunofluorescence staining. As shown in Figure 8, infection with AdE4⁺ increased Cx40 protein expression ≈ 3 -fold compared with the control (PBS) or AdE4⁻-treated mice. The levels of Cx43 expression decreased $\approx 50\%$ in heart tissue after AdE4⁺ infection as compared with control or AdE4⁻ treatment. These data were consistent with our observations of the AdE4⁺ effects on ECs in vitro. This AdE4⁺ mediation of Cxs in the heart tissue of mice indicates that AdE4⁺ also modulates Cx expression in vivo.

Discussion

Proper expression of Cxs has important implications for the maintenance of vascular homeostasis and the pathophysiology of vascular disorders.⁸ In the present study, we demonstrate for the first time that AdE4⁺ infection results in upregulation of Cx40 expression and downregulation of Cx43 expression in HUVEC cultures and in mouse heart tissue, and we show that AdE4⁺ modulates Cx expression predominantly through activation of PKA and PI3K signaling pathways.

We show inhibition of either PKA or PI3K blocked AdE4⁺-associated induction of Cx40 expression and suppression of Cx43 expression. This effect is consistent with our observation that AdE4⁺-mediated activation of PKA and PI3K/Akt promoted EC survival, in part through modulation of Cx expression. Because AdE4⁺ activation of the PI3K signaling cascade has been implicated in the increased survival of ECs, Cx40 contains phosphorylation sites for PKC-, cGMP-, or cAMP-dependent protein kinase.²⁴ Cx modulation may be a major downstream target for EC survival. The phosphorylation or dephosphorylation of Cx proteins provides a key mechanism for regulating the function of Cx junction channels.²⁵

Although Akt and PDK-1 contain PH domains and consensus kinase domains closely related to that of PKA,²⁶ in the present study, the PKA inhibitor H89 did not significantly inhibit AdE4⁺ induction of Akt phosphorylation, and activation of PKA with forskolin or cholera toxin did not induce Akt phosphorylation in ECs. Therefore, there is no evidence of a causal cross-talk between the 2 modulatory pathways, PKA and PI3K. However, both H89 and LY294002 completely abolished the effect of AdE4⁺ upregulation of Cx40 and downregulation of Cx43, implicating both PKA and PI3K signaling cascades in a common mechanism of Cx regulation. It is possible that PKA and PI3K act through a common downstream pathway, such as GSK-3 activation, to control endothelial cellular function through Cxs. Indeed, we previously demonstrated that AdE4⁺ increases pGSK-3 in ECs.²

AdE4⁺ infection of ECs alters the balance of Cx expression by downregulating Cx43 while upregulating Cx40 expression in ECs and mouse heart tissue. The mechanism for this shift may be mediated through differential activation of transcription factors. Our Northern blot

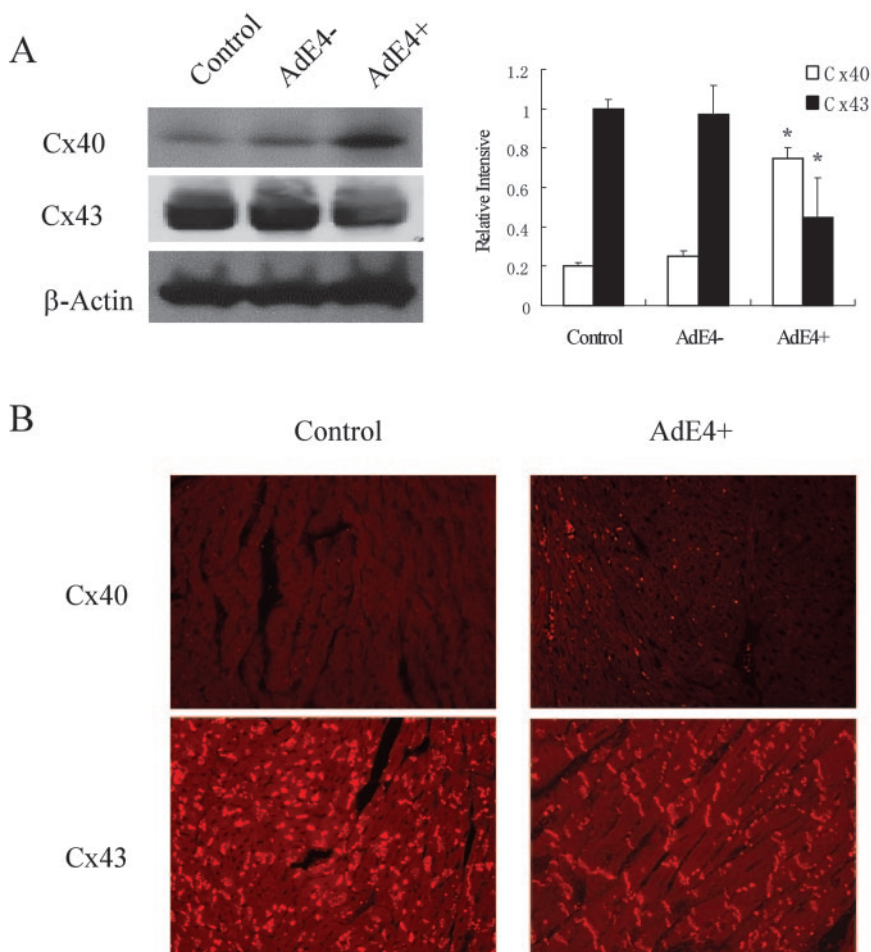


Figure 8. Cx40 and Cx43 expression in the mice heart at after intratracheal administration of AdE4⁺. A, Left, Western blot analyses were performed in mouse heart tissue after intratracheal administration of PBS (control), AdE4⁻ (10⁹ particles/mice), or AdE4⁺ (10⁹ particles/mice) for 5 days; equal amounts of whole-cell extracts of heart were resolved by SDS-PAGE and immunoblotted with Cx40, Cx43, or β -actin antibodies. Right, Band of Western blot of Cx40 and Cx43 intensities were scanned, and relative Cx40 and Cx43 protein levels were determined by normalization to the β -actin quantified by densitometry. Results represent mean \pm SE of 3 separate experiments. * $P < 0.05$ compared with control. B, After intratracheal administration of control or AdE4⁺ for 5 days, mouse heart sections were stained with anti-Cx40, anti-Cx43 antibodies. Left column, control heart; right column, heart after intratracheal administration of AdE4⁺. Results shown represent 1 experiment from a total of 3 experiments.

data suggest that AdE4⁺ selectively induces Cx40 and suppresses Cx43 gene expression at the transcriptional level. CREB is a transcription factor that is activated by phosphorylation and is the target of a variety of signaling pathways that mediate cellular responses to extracellular stimuli.²⁷ Phosphorylation of CREB at Ser133 promotes recruitment of the transcriptional coactivator CREB-binding protein (CBP),²⁸ which mediates transcriptional activation through its association with RNA polymerase II (Pol II) complexes and through intrinsic histone acetyltransferase activity. Our data showed that AdE4⁺ increased both PKA activity and CREB DNA-binding. Regulation of Cx expression modulated by AdE4⁺ was blocked by treatment with PKA inhibitor, suggesting that AdE4⁺ is a potent activator of CREB and may play an important role in regulation of connexin gene expression.

Activation of PKA is required before or during Cx protein assembly.²⁹ It has been also reported that human Cx40 gap junction channels are modulated by cAMP.³⁰ However, our results showed that intracellular cAMP induction by forskolin or cholera toxin did not affect AdE4⁺-mediated modulation of Cxs. One possible explanation may be related to the timing of treatment with forskolin or cholera toxin. The onset of action for forskolin and cholera toxin is rapid but transient, whereas Cx40 has a relatively slow onset of induction when treated with

AdE4. The temporary effects of these 2 agents may not have been observed at the 2-day time point. Alternatively, PKA activation by AdE4⁺ may be independent of cAMP activation. Indeed, PKA can be regulated upstream by cAMP-independent signaling. For example, it has been reported that phosphorylation of NF- κ B p65 may be regulated via a cAMP-independent pathway.³¹ Moreover, the PKA inhibitor H89 is also a potent selective blocker of both β_1 adrenergic receptor (β_1 AR) and β_2 adrenergic receptor (β_2 AR).³² Both of β_1 AR and β_2 AR stimulate the classic G_s-adenylyl cyclase-3',5'-adenosine monophosphate (cAMP) protein kinase A (PKA) signaling cascade.³³ Therefore, activation of β -adrenergic receptors may also involve a cAMP-independent mechanism in the regulation of Cx expression.

Cx40 is transcribed in an endothelial cell-type specific manner,³⁴ and seems to be necessary to maintain cell surface molecules involved in the differentiation and function of these cells.^{11,35} Absence of endothelial Cx40 expression leads to vasodilatation and dysfunction in the microcirculation.³⁶ Cx40 knock-in into Cx43-deficient mice rescued the subjects from the postnatal lethality of Cx43 deficiency.³⁷ Therefore, differential regulation of Cx expression and modulation of gap junction communication by AdE4⁺ may be necessary for maintenance of the function of ECs and prolongation of EC survival.

In conclusion, the present study demonstrated that AdE4⁺ regulated Cx40 and Cx43 gene expression in ECs and mouse heart tissue. PI3K and PKA are the essential signaling pathways responsible for AdE4⁺-mediated Cx expression. The significance of the finding that AdE4⁺ modulation of Cx expression may have relevance for understanding the pathophysiological mechanisms of adenovirus-related cardiovascular diseases, and strategies to regulate Cxs may provide a novel means to diminish vascular toxicities associated with E4⁺ adenoviral use.

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