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Figure legends

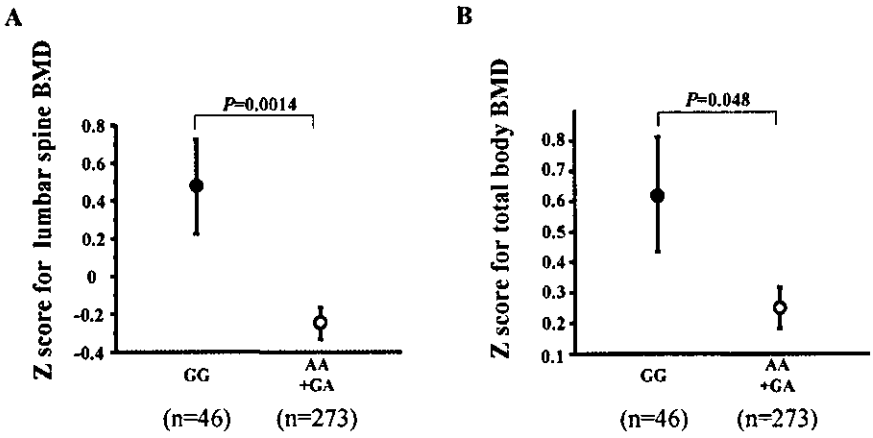
Figure 1. Z scores of lumbar spine and total body BMD in subject groups with each genotype of *ALOX15* gene polymorphism in the 5'-flanking region (-5229G/A). **A** Z scores for lumbar spine BMD are shown for genotype AA+ GA and genotype GG. Values are expressed as mean ± SE. Numbers of subjects are shown in parentheses. **B** Z scores for total body BMD are shown in the same manner as in **A**.

Table 1. Comparison of background, BMD and biochemical data between subjects bearing at least one A allele (AA + GA) and subjects with no A allele (GG) in the *ALOX15* gene 5'-flanking region (-5299G/A).

Items	Genotype (mean ± SD)		*P* value
	GG	GA+AA	
Number of subjects	46	273	
Age (years)	69.0 ± 8.9	66.3 ± 8.9	NS
Height (cm)	149.5 ± 6.9	150.3 ± 6.1	NS
Body weight (kg)	49.6 ± 8.6	50.3 ± 7.9	NS
Lumber spine BMD (Z score)	0.48 ± 1.70	-0.25 ± 1.37	0.0014
Total body BMD (Z score)	0.62 ± 1.12	0.25 ± 1.01	0.048
ALP (IU/L)	185.6 ± 63.9	193.4 ± 66.0	NS
I-OC (ng/mL)	8.2 ± 3.2	7.8 ± 3.6	NS
DPD (pmol/μmol of Cr)	7.0 ± 3.0	7.6 ± 2.7	NS
Intact PTH (pg/mL)	38.6 ± 20.0	35.2 ± 15.1	NS
Calcitonin (pg/mL)	16.6 ± 4.5	23.1 ± 11.4	NS
1,25 $(OH)_2D_3$ (pg/ mL)	33.0 ± 7.7	35.7 ± 11.8	NS
TC (mg/dL)	193.0 ± 45.0	199.4 ± 36.5	NS
TG (mg/dL)	142.5 ± 74.0	142.4 ± 81.8	NS
% fat	32.1 ± 6.6	31.9 ± 7.7	NS
BMI	22.1 ± 3.0	22.1 ± 3.1	NS

BMD, bone mineral density; ALP, alkaline phosphatase; I-OC, intact-osteocalcin; DPD, deoxypyridinoline; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; BMI, body mass index; NS, not significant. Statistical analysis was performed according to the method described in the text.

Fig. 1.


A
Z score for lumbar spine BMD
P=0.0014
0.8
0.6
0.4
0.2
0
-0.2
-0.4
GG
AA
+GA
(n=46)
(n=273)
B
Z score for total body BMD
P=0.048
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
GG
AA
+GA
(n=46)
(n=273)

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17β-Estradiol inhibits cardiac fibroblast growth through both subtypes of estrogen receptor

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Abstract

The effect of 17β-estradiol (E2) on the proliferation of cardiac fibroblasts (CFs) remains controversial. This study investigated which subtype of estrogen receptor (ER), ERα or ERβ, mediated the effect of E2 on CF growth by the gain of function analysis using an adenovirus vector. One hundred nanomoles per liter of E2 attenuated DNA synthesis by up to 10%, and transactivated the estrogen-responsive element determined by luciferase assay in rat neonatal CFs. We constructed replication-deficient adenoviruses bearing the coding region of human ERα, ERβ, or the dominant-negative form of ERβ (designated AxCAERα, AxCAERβ, and AxCADNERβ, respectively). When CFs were infected with AxCAERα or AxCAERβ at multiplicity of infection of 20 or higher, DNA synthesis was decreased by 50% in response to E2 and the effect was abolished by co-infection with AxCADNERβ. Similarly, transcriptional activity of ER in CFs infected with AxCAERα or AxCAERβ was markedly enhanced and co-infection with AxCADNERβ abolished the effects. These results suggest that E2 inhibits CF growth and that both ER subtypes mediate the effect comparably and redundantly.
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Keywords: Cardiac remodeling; Cardiac fibroblast; Hormones; Receptor; Adenovirus

Structural remodeling of the ventricular wall takes place in several cardiac disorders including acute myocardial infarction, cardiomyopathy, and hypertensive heart disease. Histopathologically, it is characterized by a structural rearrangement of components of the normal chamber wall that involves cardiomyocyte hypertrophy, proliferation of cardiac fibroblast (CFs), fibrosis, and cell death [1]. In the adult heart, CFs substantially constitute the non-myocyte cells [2] and contribute to cardiac remodeling by undergoing proliferation, depositing extracellular matrix proteins which are mainly produced by CFs in the myocardium, and eventually replacing myocytes with fibrotic scar tissue. CFs also produce matrix metalloproteinases, growth factors, and cytokines, all of which are involved in the maintenance of myocardial structure, and in diseased hearts play pivotal roles in remodeling [3]. Recent studies have shown that the interactions between CFs and cardiomyocytes are essential for the progression of cardiac remodeling [3]. Thus, it is clinically important to inhibit CF growth in the process of cardiac remodeling.

From several epidemiological studies, estrogen (E2) is thought to have a protective effect against left ventricular hypertrophy which is an important cardiovascular risk factor for morbidity and mortality [4–6]. Premenopausal women have a lower prevalence of left ventricular hypertrophy than their age-matched male counterparts [4]. Left ventricular mass is significantly greater in men than in women even after indexing for body surface area [5,6]. Experimental studies have shown cardioprotective roles of E2 [7–10], however, the direct effect of E2 on cardiac cell growth remains to be determined. Previous studies have demonstrated that the

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exogenous administration of E2 either decreased [7], increased [11], or had no effect on DNA synthesis in cultured CFs [12,13].

Most biological effects of E2 are mediated by the estrogen receptor (ER). ER has two subtypes, classical ERα and newly identified ERβ [14]. It is reported that both ER subtypes are expressed in CFs [13,15]. However, little is known about the involvement of ER in CF growth, although many transcriptional factors including nuclear receptors regulate the functions of CFs in the process of cardiac remodeling [3]. There is only one report showing that the inhibitory effect of E2 on CF growth is independent of ER [16]. Adenovirus-mediated gene transfer is a useful tool to clarify the precise role of a specific gene. We constructed replication-deficient adenovirus vectors carrying ERα, ERβ or dominant-negative form of ERβ. In this study, to determine the effect of E2 on CF growth and which ER subtype plays a pivotal role in the cell growth, we evaluated DNA synthesis in CFs overexpressing each ER subtype using adenovirus vector. Here we show that E2 attenuated DNA synthesis by up to 10% in rat neonatal CFs and that adenovirus-mediated overexpression of either of the ER subtypes in CFs augmented growth inhibition in a ligand-dependent manner.

Methods

Cell culture. Rat CFs were harvested from the heart of Wistar neonatal rats at birth, as previously reported by Zang et al. [17] Briefly, the hearts were removed from neonatal rats and minced with scissors until very small pieces were produced. The pellet of minced tissue was then resuspended in 1% collagenase and incubated at 37 °C for 2 h. Next, the tissue was resuspended in 0.25% trypsin and incubated at 37 °C for 2 h. The digested tissue was resuspended in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), 25 mM Hepes (pH 7.4), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Twenty-four hours later, the medium was aspirated and the fresh medium was added. CFs at 6–9 passages were used in the experiments. At the time of experiments, we used dextran-coated charcoal-stripped FBS (DCC-FBS) and phenol-red-free M199 medium to avoid contamination with steroids or estrogen receptor agonists.

Construction of adenovirus vectors carrying estrogen receptor subtypes and transfer into CFs. Replication-deficient adenovirus vectors carrying the CMV-IE enhancer, chicken β-actin promoter, and the coding region of human ERα, ERβ, or dominant-negative form of ERβ, were constructed by use of adenovirus expression vector kit (Takara Shuzo, Kyoto, Japan) as described before [18] and named AxCAERα, AxCAERβ, and AxCADNERβ, respectively. CFs were exposed to different multiplicities of infection (MOI) of either AxCAERα, AxCAERβ, AxCADNERβ, or a replication-deficient recombinant adenovirus carrying the *Escherichia coli* β-galactosidase gene (AxCALacZ) for 2 h in DMEM with 5% FBS. Then, the cells were rinsed with phosphate-buffered saline once and used for the experiments.

RNA isolation, reverse transcription polymerase chain reaction. Total RNA was prepared from CFs and rat ovary as positive control, using Isogen (Wako Pure Chemical Industries, Osaka, Japan). Then, 1 μg total RNA was reverse transcribed into cDNA and one-twentieth of the product was amplified for 35 cycles. Negative control reverse transcription polymerase chain reactions (RT-PCRs) were performed by omitting reverse transcriptase. The primer pairs used in PCR are: CTAAGAAGAATAGCCCCGCC (forward, +1126 to +1145) and CAGACCAGACCAATCATCAGG (reverse, +1402 to +1382) for rat ERα (GenBank Accession No. NM_012689), and CGACTGAGCAC AAGCCCAAATG (forward, +76 to +97) and ACGCCGTAA TGATACCCAGATG (reverse, +353 to +332) for rat ERβ (GenBank Accession No. AB012721).

Measurement of [^{3}H]thymidine incorporation. CFs seeded onto 24-well tissue culture plates were grown until 70–90% confluent and then made quiescent by culturing in phenol-red-free M199 medium (Gibco) for 24 h. Then, the cells were stimulated with 5% DCC-FBS in the presence of water-soluble 17β-estradiol (Sigma–Aldrich, Japan) for 24 h, followed by pulse-labeling with 1 μCi/ml [^{3}H]thymidine for 3 h. [^{3}H]Thymidine incorporated into DNA was determined as previously described [19].

Number of CFs. CFs were seeded onto six-well multiplates and cultured until a confluent state was obtained. After infection of CFs with adenovirus vectors, the medium was replaced with phenol-red-free M199 to arrest the growth. After 24 h, the medium was replaced again with phenol-red-free M199 containing 5% DCC-FBS with E2 or vehicle. After incubation for 48 h, the cells were trypsinized and suspended. Then the number of cells was determined using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL).

Luciferase assays. CFs were transfected with ERE-TK-Luc reporter plasmid and pRL-SV40 control plasmid using FuGENE6 (Roche) for 24 h according to the manufacturer's instructions [20]. Then, CFs were incubated in phenol-red-free M199 medium with 1% DCC-FBS for 24 h and exposed to E2 for additional 24 h. We measured two kinds of luciferase activity using a dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol, and the ratio of firefly luciferase activity to that of *Renilla* luciferase in each sample was used as a measure of normalized luciferase activity [20].

Western blotting. After infection with adenovirus vector, cells were incubated with serum-free M199 medium for 24 h to detect ER subtypes. Cells were washed quickly with phosphate-buffered saline twice and lysed in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors cocktail; Complete, Mini; Roche). The samples were separated on 12% SDS–PAGE, electroblotted onto nitrocellulose membrane, and immunoblotted with anti-ERα polyclonal antibody (H-184; Santa Cruz, 1:1000 dilution), anti-ERβ monoclonal antibody (CWK-F12, kindly provided by Dr. Benita S. Katzenellenbogen, thanks and details are given in Acknowledgements, 1:1000 dilution). Antibody was detected with a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Life Science).

Statistical analysis. The dose–response effect of E2 or ER overexpression on DNA synthesis in CFs was analyzed using one-way factor ANOVA. If a statistically significant effect was found, Newman–Keuls test was performed to isolate the difference between the groups. A value of $P < 0.05$ was considered statistically significant. All data in the text and figures are expressed as means ± SE.

Results

Endogenous expression of ER subtypes and the effect of E2 on CF growth

To investigate the endogenous expression of ER in rat CFs, RT-PCR amplification was performed. Both rat ERα and ERβ were expressed in CFs (Fig. 1A). At physiological concentrations, E2 inhibited the proliferation of CFs dose-dependently by up to 10% (Fig. 1B).

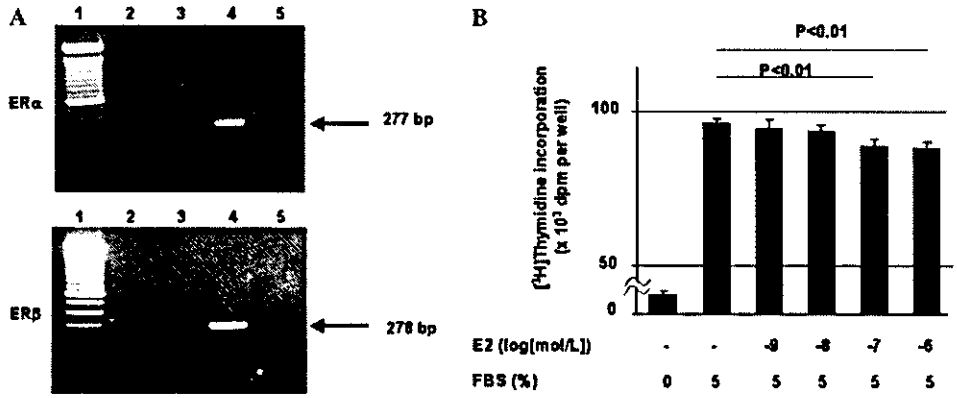

Fig. 1. ER gene expression and the effect of E2 on rat CF proliferation. (A) RT-PCR was performed using the cDNA of rat ovary as a positive control with (lane 4) or without (lane 5) reverse transcriptase and using the cDNA of rat CFs with (lane 2) or without (lane 3) reverse transcriptase. Lane 1 shows the molecular weight marker. B) Serum-starved CFs were stimulated with 5% DCC-FBS in the absence or presence of 10–1000 nmol/L 17β-estradiol for 24 h. [^{3}H]Thymidine incorporation into DNA was determined by pulse-labeling for the last 3 h of incubation. Results are shown as means ± SE ($n = 3$). Similar results were obtained in three independent experiments.

Expression of ER subtype in CFs by adenovirus-mediated transfer of the ER subtype genes

Expression of the ERα and ERβ protein was confirmed by Western blot analysis (Fig. 2A). Although both ER subtypes were detected by RT-PCR, the protein expression was undetectable in non-transfected CFs; the bands corresponding to ERα (65 kDa) or ERβ (55 kDa) were seen in CFs infected with AxCAERα, or with AxCAERβ, respectively (Fig. 2A), and also in MCF-7 cells or rat ovary which were used as positive controls (data not shown). The protein expression was increased by overexpression MOI-dependently. We also checked the protein level of both ER subtypes in non-infected cells after the addition of E2. However, E2 did not induce the protein of either ER subtype in our experimental conditions (data not shown).

Effect of adenovirus-mediated transfer of the ER subtype genes on CF growth

When AxCALacZ was introduced into CFs at more than 60 MOI, DNA synthesis reduced in a MOI-dependent manner in the absence of E2 (data not shown). Therefore, we examined DNA synthesis at 60 MOI or less to avoid the influence of adenovirus itself on DNA synthesis. CFs infected with AxCALacZ showed no additional decrease in DNA synthesis in response to E2 (Fig. 2B). In contrast, when CFs were infected with AxCAERα or AxCAERβ at more than 10 MOI, DNA synthesis was significantly inhibited in a MOI-dependent manner in response to E2 to grossly similar extent. To confirm this, the cell number was counted in the presence or absence of E2. Comparable to the thymidine incorporation assay, overexpression of either ERα or ERβ enhanced the inhibitory effect of E2 on CF growth (Fig. 2C). Moreover, in CFs infected with either AxCAERα or AxCAERβ at 20 MOI, E2 decreased DNA synthesis in a concentration-dependent manner at 10^{-11}–10^{-6} mol/L (Fig. 3). Taking these results together, the effects of AxCAERα and AxCAERβ seemed comparable. To examine whether the effect of ER transfer is truly ER subtype dependent, we investigated DNA synthesis in CFs co-infected with AxCAERα or AxCAERβ and AxCAERDNβ. The reduction of DNA synthesis in CFs infected with AxCAERα or AxCAERβ alone at 20 MOI was abolished by co-infection with AxCAERDNβ (Fig. 4).

Transcriptional activity of ERE in CFs infected with ER genes

We examined the transcriptional activity of ER by luciferase activity of the ERE reporter plasmid. In non-infected CFs, 100 nmol/L E2 augmented the luciferase activity of ERE by approximately 1.3-fold compared to vehicle ($p = 0.02$) (Fig. 5). CFs infected with AxCAERα or AxCAERβ at 20 MOI showed a strong increase in transcriptional activity in the presence of E2; 3.3-fold increase with AxCAERα and 3.9-fold increase with AxCAERβ in response to E2. This increase was completely abolished by co-infection with AxCADNERβ.

Discussion

Conflicting results have been reported concerning the effect of E2 on CF growth. One group demonstrated that CF growth was not affected by E2 [12]. Two groups showed that E2 inhibited CF growth [7,13], whereas another has shown that E2 enhanced CF growth through mitogen-activated protein kinase-dependent pathway [11]. Thus, the effect of estrogen on CF growth remained to be addressed. In this study, E2 inhibited DNA synthesis in CFs by up to 10%, and this inhibition was augmented by overexpression of either of ER subtypes,

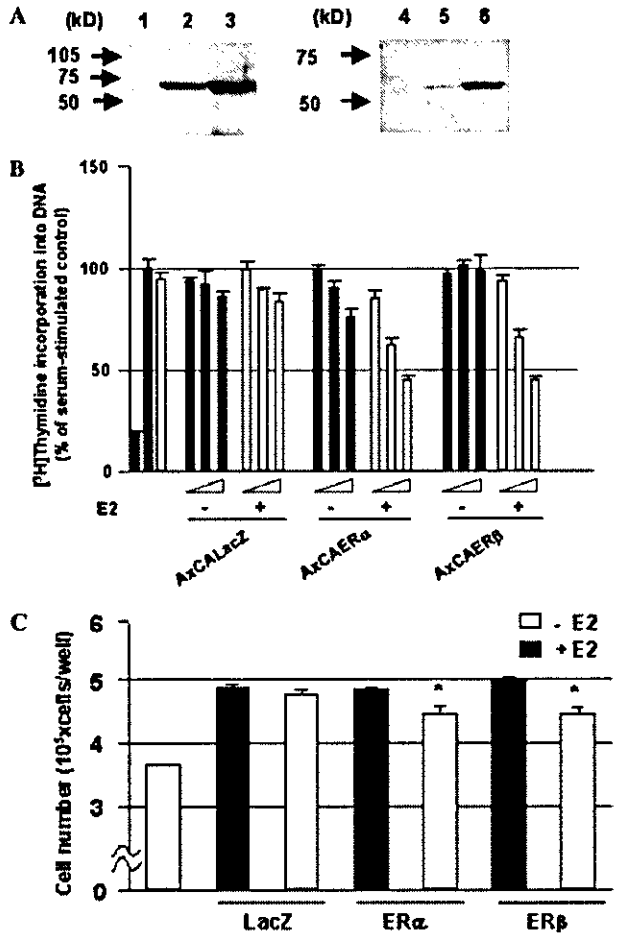

Fig. 2. Induction of ER protein and inhibition of CF growth by adenovirus-mediated transfer of ER genes. (A) CFs were infected without (lanes 1 and 4), or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or 10 and 100 MOI of AxCAERβ (lanes 5 and 6, respectively). Western blot analysis was performed with 40 μg of protein per lane by using an anti-ERα polyclonal antibody (left panel) or anti-ERβ monoclonal antibody (right panel). CFs seeded onto a 24-well plate (B) or 6-well plate (C) were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (1, 10, and 30 MOI (B), respectively, from left to right, or 30 MOI (C)) for 2 h and serum-deprived for 24 h. [³H]Thymidine incorporation into DNA (B) was determined at 24 h after the stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/L E2 and presented as a percentage of the serum-stimulated control. The left-sided 3 lines indicate non-infected CFs with serum-free medium, 5% DCC-FBS in the absence of E2, and 5% DCC-FBS in the presence of 100 nmol/L E2, respectively (B). Cell numbers were counted after 48 h of stimulation with 5%DCC-FBS in the presence or absence of 100 nmol/L E2 (C). The left-sided line indicates non-infected CFs before the stimulation. $^*P < 0.01$ vs CFs without E2. Results are shown as means ± SE ($n = 3$) (B,C). Similar results were obtained in three independent experiments.

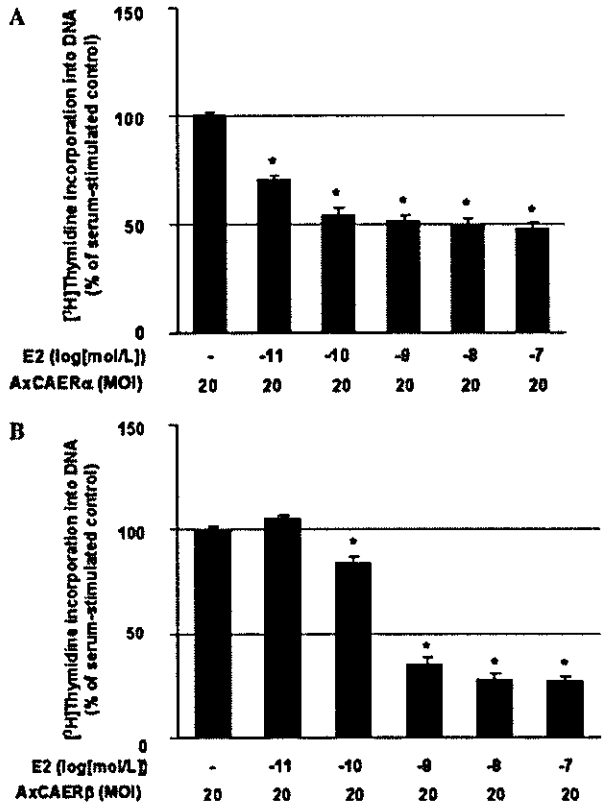

Fig. 3. Dose–response effect of E2 on DNA synthesis in CFs overexpressing ER subtypes. CFs seeded onto a 24-well plate were exposed to DMEM containing 10 MOI of AxCAERα (A) or AxCAERβ (B) for 2 h, and were serum-deprived for 24 h. [³H]Thymidine incorporation into DNA was determined at 24 h after the stimulation with 5% DCC-FBS in the absence or presence of the indicated concentrations of E2 and presented as a percentage of CFs without E2. $^*P < 0.01$ vs CF without E2. Results are shown as means ± SE ($n = 4$). Similar results were obtained in three independent experiments.

indicating that both ER subtypes work to inhibit CF growth in a redundant fashion.

Both ER subtypes are expressed in cardiac myocytes and CF as shown by Western blotting [13,15,21], and are transcriptionally active [15], suggesting that ER subtypes play a role in cardiac cells. Moreover, it is reported that the expression of ER subtypes in cardiac cells was regulated by physiological or pathophysiological stimuli such as E2 [15] and hypoxia [22]. Protein levels of both ER subtypes were increased in CFs and cardiac myocytes in response to E2 [15]. Under hypoxic condition, the protein level of ERβ but not of ERα was upregulated while the presence of E2 decreased the level of ERβ protein in CFs [22]. Modulation of ER subtype expression by E2 was not confirmed in the present study (data not shown) presumably because the expression in non-transfected CFs was too low to detect by Western blotting. Changes of ER expression in cardiac cells associated with cardiovascular disease are currently unknown. However, the gain-of-function analysis implies the physiological relevance by mimicking the conditions of the previous reports [13,15,21,22]. Another rationale in using the overexpression system was to compare the effects on CF growth between ER subtypes. Because adenovirus vectors successfully induced ER subtypes to a similar extent, we could interpret the results clearly.

Several reports have examined the role of ER subtypes in proliferation using the gene transfer techniques into cell lines [23–26]. Cheng and Malayer [26] have reported that overexpression of ERα in an ER-negative

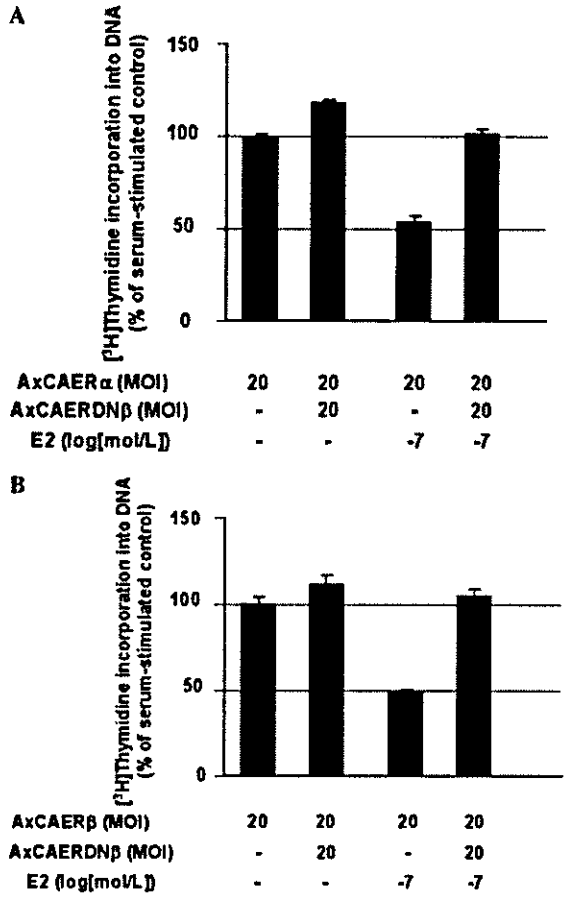

Fig. 4. The effect of dominant negative ER on ER subtype overexpression in CFs. CFs seeded onto a 24-well plate were exposed to DMEM containing 20 MOI of AxCAERα (A) or AxCAERβ (B) and the indicated MOI of AxCADNERβ. After infection, CFs were serum-deprived for 24 h. [³H]Thymidine incorporation into DNA was determined at 24 h after the stimulation with 5% DCC-FBS in the absence or presence of 100 nmol/L of E2, and were presented as a percentage of CFs infected with AxCAERα alone (A) or AxCAERβ alone (B) without E2. Results are shown as means ± SE ($n = 3$). Similar results were obtained in three independent experiments.

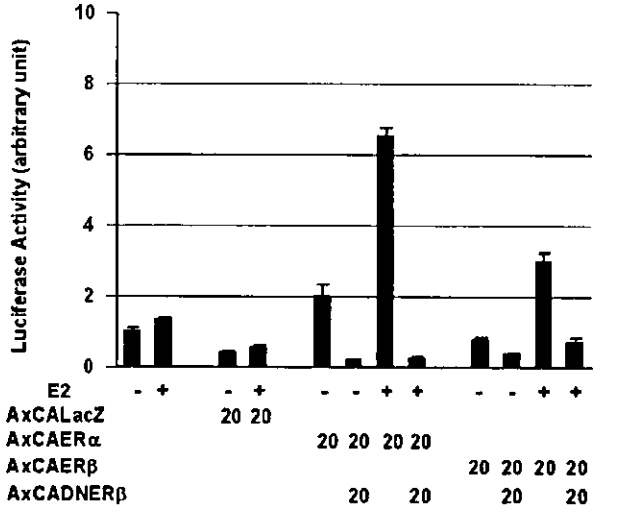

Fig. 5. The influence of ER overexpression on the promoter activity of ER responsive enhancer elements in CFs. CFs were infected with AxCALacZ, AxCAERα, AxCAERβ, or AxCADNERβ at the indicated MOI for 2 h, and transfected with the luciferase reporter plasmids containing ERE and the pRL-SV40 control plasmid. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/L E2 for 24 h. Results are shown as means ± SE ($n = 3$). Similar results were obtained in three independent experiments.

rat fibroblast cell line, rat-1, resulted in an estrogen-dependent small (<10%) but significant increase in cell proliferation but overexpression of ERβ did not affect proliferation. In contrast, Lazennec et al. [24] have shown that overexpression of ERα in an ER-negative human breast cancer cell line, MDA-MB-231, led to a hormone-dependent inhibition of proliferation, whereas overexpression of ERβ caused a hormone-independent inhibition. Taken these results together with other reports examining the effect of ER overexpression in non-CF cells [23–25], the role of ER subtypes in cell proliferation may be different between cell types. This may also be the case with our results and the results in fibroblasts by Cheng and Malayer [26]. We used neonatal primary cultured CFs that expressed low levels of both ER subtypes, while Cheng et al. used a cell line derived from embryo fibroblasts that did not express ER.

The divergent roles of ER subtypes can be explained by the differential induction of estrogen response genes, the different interactions with promoter elements including AP-1 sites [27] and SP-1 sites [28] in an ERE-independent manner, or differential recruitment of transcriptional co-factors. In the present study, however, ERα and ERβ inhibited CF growth and transactivated the ERE similarly in response to E2. The only difference between ER subtypes observed in this study was that overexpression of ERα exerted the effects on cell growth and transcriptional activity ligand-independently (Figs. 2B and 5), although these effects were slight and might be non-specific. Accordingly, it is suggested that ERα and ERβ mediate the inhibitory effect of E2 on CF growth in a redundant or compensatory fashion as is the case with some gene superfamilies [29,30].

Our findings provide a mechanistic insight into the understanding of how E2 acts in CFs in the process of cardiac remodeling. Our data imply that the proliferation of CFs involved in cardiac hypertrophy and fibrosis can be inhibited by E2 as is shown in clinical and experimental settings [4–10], and that both ER subtypes expressed in CFs mediate the inhibitory effects of E2. Unfortunately, recent clinical trials [31,32] have failed to show beneficial effects of hormone replacement therapy on cardiovascular disease. Alternatively, specific ligands such as selective ER modulators [33] might exert beneficial clinical effects, particularly in combination with the gene transfer of ER subtypes, to inhibit cardiac remodeling. To test this possibility, in vivo experiments using ER overexpression and selective ER modulators should be performed in the future.

Acknowledgments

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Estrogen receptor β mediates the inhibitory effect of estradiol on vascular smooth muscle cell proliferation

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Abstract

Objectives: It has been demonstrated that 17β-estradiol (E2) has an inhibitory effect on the proliferation of vascular smooth muscle cells (VSMCs) through an estrogen receptor (ER)-dependent pathway. Both ER subtypes, classical ER (ERα) and the newly identified ER subtype (ERβ), are expressed in VSMCs. However, it remains unknown which receptor plays the critical role in the inhibitory effect on VSMC proliferation. **Methods and results:** We constructed replication-deficient adenoviruses bearing the coding region of human ERα, ERβ, and the dominant-negative form of ERβ (designated AxCAERα, AxCAERβ, and AxCADNERβ, respectively). Prior to infection with the adenoviruses, 100 nmol/l E2 attenuated DNA synthesis by up to 14% and transactivated the estrogen-induced expression of the desired mRNA in rat VSMCs. This was accompanied by increased transcriptional activity of estrogen responsive element in response to E2, and the increase was comparable between AxCAERα and AxCAERβ. When VSMCs were infected with AxCAERβ at a multiplicity of infection of 5 or higher, DNA synthesis as well as cell number decreased by 50% in response to E2, and the effect was abolished by co-infection with AxCADNERβ. In contrast, when VSMCs were infected with AxCAERα, the reduction in DNA synthesis was minimal. **Conclusions:** Our results indicate that ERβ is more potent than ERα in the inhibitory effect on VSMC proliferation.
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Keywords: Atherosclerosis; Gene expression; Hormones; Receptors; Smooth muscle

1. Introduction

The proliferation of vascular smooth muscle cells (VSMCs) is a common feature associated with vascular proliferative disorders such as atherosclerosis and restenosis after balloon angioplasty [1]. Inhibition of VSMC growth is thus one therapeutic target for the prevention of vascular diseases. Estrogen exhibits a variety of actions on the vascular wall that could be involved in its atheroprotective effects [2,3]. These include the stimulation of nitric oxide production by endothelial cells [4] and the inhibition of VSMC proliferation [5–8]. However, results from recent randomized double-blind trials, which were conducted to evaluate the effect of hormone replacement therapy (HRT) in primary prevention [9] and in secondary prevention [10], have failed to show a protective effect of HRT on cardiovascular disease. These conflicting data might result from the prothrombotic effects of estrogen [11], which could abolish the beneficial effects of estrogen on vascular function. Additionally, progestin, combined with estrogen to decrease the risk of endometrial cancer during HRT, might exert prothrombotic and proinflammat-

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ory effects. So far, the protective effects of estrogen alone on cardiovascular diseases remain unknown.

Most of the effects of estrogen are thought to be mediated by the estrogen receptor (ER), a member of the intra-nuclear receptor family. A new subtype of ER, ERβ, was discovered in 1996 [12], and has a somewhat different expression and localization patterns and transcriptional activity in reproductive and non-reproductive organs from those of classical ERα [13]. The ER subtypes may provide a clue to answering the question of why estrogen exerts differential effects in various cells and tissues; that is, estrogen stimulates proliferation in MCF-7 breast cancer cells [14] and osteoblastic cells [15], but inhibits proliferation in VSMCs. Morey et al. showed that, in VSMCs, the growth inhibitory effect of estrogen can be blocked by the nonspecific estrogen receptor antagonists tamoxifen [6] and ICI 182,780 [8]. However, it remains unknown which ER subtype mediates the growth inhibitory effect of estrogen in VSMCs, where both ER subtypes are expressed [16–18].

Also, in vivo studies using genetically engineered mice have provided insufficient information on this issue. Estrogen inhibites VSMC proliferation of the medial area in response to vascular injury in ERα knockout mice [19] as well as in ERβ knockout [20] and double knockout mice [21]. In contrast, estrogen has no detectable effect on VSMC proliferation in fully null ERα knockout mice [22], suggesting that a splice variant of the ERα gene in the previous knockout mice lines plays a role. However, some points remain unclear in the study. Would the function of a splice variant, scarcely expressed in the vascular wall, really be as efficient as that of wild-type ERα? VSMC proliferation is inhibited in newly generated ERα knockout mice in an estrogen-independent manner as compared to wild-type mice [22]. This result suggests that ERα could exert ligand-independent VSMC proliferation, an interesting, but not established, concept.

In the rat carotid injury model, ERβ is predominantly expressed after injury [23], and the isoflavone phytoestrogen genistein, which showed a 20-fold higher binding affinity to ERβ than to ERα, exhibited a vasculoprotective effect. Taken together, ERβ might be a main mediator for the estrogen-mediated vasculoprotective effect. In the present study, to clarify which ER subtype plays the pivotal role in the inhibitory effect of estrogen on VSMC proliferation, we used adenovirus vectors to transfer ER subtypes into VSMCs. As reported previously, estradiol (E2) attenuates DNA synthesis dose-dependently. Adenovirus-mediated overexpression of ERβ in VSMCs augments growth inhibition in a ligand-dependent manner.

2. Methods

2.1. Cell culture

Rat VSMCs were harvested from the aortae of 8-week-old Wistar male rats by enzymatic dissociation according to the modified method of Chamley et al. [24]. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo. Human aortic VSMCs were purchased from Clonetics (Cat. #CC-2571). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY, USA), 25 mM HEPES (pH 7.4), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Rat VSMCs at six to 10 passages were used in the experiments. At the time of the experiments, we used dextran-coated charcoal-stripped FBS (DCC-FBS) and phenol red-free RPMI1640 medium for rat VSMCs and M199 medium for human VSMCs to avoid contamination with steroids and estrogen receptor agonist. All dishes used in this study were purchased from Asahi Techno Glass Co., Ltd., Tokyo.

2.2. Construction of adenovirus vector carrying estrogen receptor subtypes and transfer into VSMCs

Replication-deficient adenovirus vectors carrying the CMV-IE enhancer, chicken β-actin promoter, and the coding region of human ERα, ERβ, or the dominant-negative form of ERβ [25] were constructed by use of an adenovirus expression vector kit (Takara Shuzo Co., Kyoto, Japan) as described before [26], and are denoted AxCAERα, AxCAERβ, and AxCADNERβ, respectively. VSMCs were exposed to different multiplicities of infection (MOI) of either AxCAERα, AxCAERβ, AxCADNERβ, or a replication-deficient recombinant adenovirus carrying the *Escherichia coli* β-galactosidase gene (AxCALacZ) for 2 h in DMEM with 5% FBS. The cells were then rinsed with phosphate-buffered saline once, and used for the experiments.

2.3. RNA isolation, reverse transcription polymerase chain reaction (RT-PCR), and Northern blot analysis

For RT-PCR, total RNA was prepared from VSMCs and, as a positive control, rat ovary, using Isogen (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, 1 μg total RNA was reverse transcribed into cDNA, and 1/20 of the product was amplified for 35 cycles. Negative control RT-PCR reactions were performed by omitting reverse transcriptase. The primer pairs used in PCR were: CTAAGAAGAATAGCCCCGCC (forward, +1126 to +1145) and CAGACCAGACCAATCATCAGG (reverse, +1402 to +1382) for rat ERα (GenBank, accession number NM_012689), and CGACTGAGCACAAGCCCAAATG (forward, +76 to +97) and ACGCCGTAATGATACCCAGATG (reverse, +353 to +332) for rat ERβ (GenBank, accession number AB012721). Both PCR products were subsequently sequenced, and were used as the probes for rat ERα and ERβ.

For Northern blotting, VSMCs were plated on 10 cm diameter dishes, and infected with adenovirus bearing either ER subtype at 70–90% confluence. At 24 h after infection, VSMCs were harvested using ISOGEN. The RNA was fractionated on 1.3% formaldehyde-agarose gel and transferred to nylon filters (Hybond-N; Amersham Life Science Inc.). The filters were hybridized at 68 °C for 2 h with a random-primed ^{32}P-labeled human ER cDNA probe in QuikHyb solution (Stratagene) and autoradiographed. The products digested by EcoRI and PVUII from human ERα plasmid and EcoRI from human ERβ plasmid were used as the human ERα and human ERβ probe, respectively.

2.4. Western blot analysis

Cells were washed quickly with phosphate-buffered saline twice, and lysed in RIPA buffer: 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors cocktail (Complete, Mini; Boehringer Mannheim). The samples were separated on 12% SDS–PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with anti-ERα polyclonal antibody (H-184; Santa Cruz, 1:1000 dilution), anti-ERβ monoclonal antibody, CWK-F12 (kindly provided by Dr. Benita S. Katzenellenbogen, Department of Molecular and Integrative Physiology, University of Illinois College of Medicine, 1:1000 dilution), or cyclin A polyclonal antibody (C-19; Santa Cruz, 1:1000 dilution). Antibody was detected with a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Life Science Inc.).

2.5. Transfection and luciferase assays

We used the ERE-TK-Luc reporter plasmid and a firefly luciferase reporter vector as previously described [24]. VSMCs were transfected with ERE-TK-Luc reporter plasmid and pRL-SV40 control plasmid using FuGENE6 (Roche) for 24 h according to the manufacturer's instructions. Then, VSMCs were incubated in phenol-red-free RPMI1640 containing 0.1% DCC-FBS for 24 h, and exposed to 1–100 nmol/l E2 (water-soluble 17β-estradiol; Sigma–Aldrich Japan), 10–1000 nmol/l ICI 182,780 or vehicle, β-cyclodextrin solution (Sigma) as a vehicle for water-soluble E2, for an additional 24 h. We measured two kinds of luciferase activity using a dual-luciferase reporter assay system (Promega) according to the manufacture's protocol, and the ratio of firefly luciferase activity to that of Renilla luciferase in each sample was used as a measure of normalized luciferase activity. Each experiment was repeated at least three times.

2.6. Measurement of [^{3}H]thymidine incorporation into DNA of VSMCs

VSMCs seeded onto 24-well tissue culture plates were grown until 70–90% confluence, and then made quiescent by culturing them in phenol-red-free RPMI1640 medium (Gibco) for 24 h. Then, the cells were stimulated with 5% DCC-FBS in the presence of E2 (water-soluble 17β-estradiol; Sigma–Aldrich Japan) or vehicle for 24 h, followed by pulse-labeling with 1 μCi/ml [^{3}H]thymidine for 3 h. [^{3}H]Thymidine incorporated into DNA was determined as previously described [5].

2.7. Number of VSMCs

VSMCs were seeded onto six-well multiplates and cultured until a confluent state was obtained. After infection of VSMCs with adenovirus vectors, the medium was replaced with phenol-red-free RPMI1640 to arrest the growth. After 24 h, the medium was replaced again with phenol-red-free RPMI1640 containing 5% DCC-FBS with E2 or vehicle. After incubation for 48 h, the cells were trypsinized and suspended. Then the number of cells was determined using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL, USA).

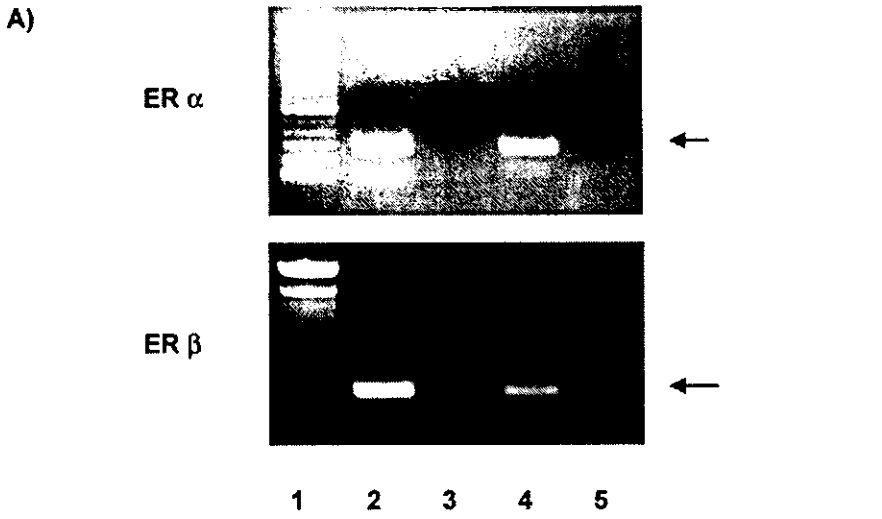
2.8. Statistical analysis

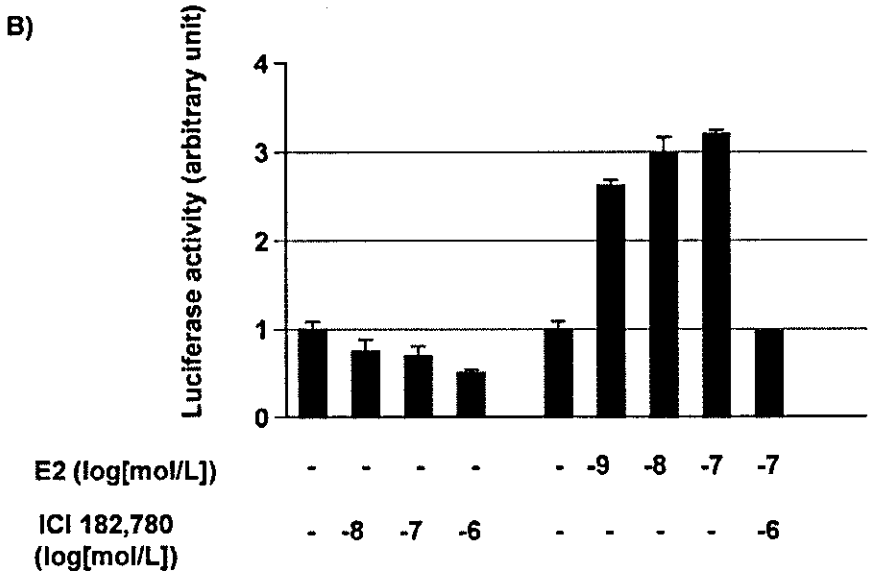
The dose–response effect of E2 or adenoviruses on DNA synthesis in VSMCs and the luciferase activity in E2-treated VSMCs were analyzed using one-way ANOVA. If a statistically significant effect was found, Newman–Keuls' test was performed to isolate the difference between groups. A value of $P<0.05$ was considered statistically significant. All data in the text and figures are expressed as mean±S.E.

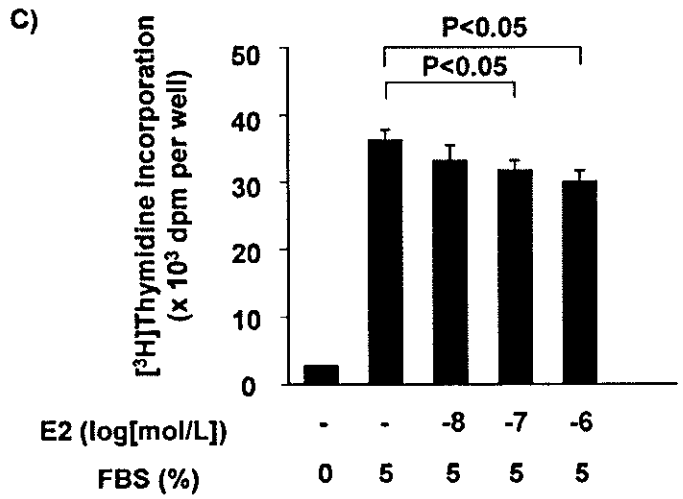
3. Results

3.1. Endogenous expression of ER subtypes in VSMCs and effect of E2 on VSMC growth

To investigate the endogenous expression of ER in rat VSMCs, RT-PCR amplification was performed. Both rat ERα and ERβ were expressed in VSMCs (Fig. 1A). Next, we examined the transcriptional activity of endogenous ER by means of the luciferase activity of the ERE reporter plasmid, and the inhibitory effect of E2 on VSMC proliferation by evaluating DNA synthesis. E2 at 1–100 nmol/l augmented the luciferase activity of ERE by approximately three-fold compared to vehicle, and this increase was abolished by the nonselective pure ER antagonist ICI 182,780 (AstraZeneca) (Fig. 1B). At these concentrations, E2 inhibited the proliferation of VSMCs dose-dependently (Fig. 1C). In the absence of E2, ICI 182,780 inhibited the luciferase activity dose-dependently by up to 50% compared to vehicle (Fig. 1B), but did not influence thymidine incorporation into VSMCs at concentrations of 10–1000 nmol/l (data not shown). This result indicates that there may be some leakage of estrogenic activity from cell


A)
ER α
ER β
1 2 3 4 5


B)
Luciferase activity (arbitrary unit)
0
1
2
3
4
E2 (log[mol/L]) - - - - - -9 -8 -7 -7
ICI 182,780 (log[mol/L]) - -8 -7 -6 - - - - -6


C)
[3H]Thymidine incorporation (x 10³ dpm per well)
P<0.05
P<0.05
0
10
20
30
40
50
E2 (log[mol/L]) - - -8 -7 -6
FBS (%) 0 5 5 5 5

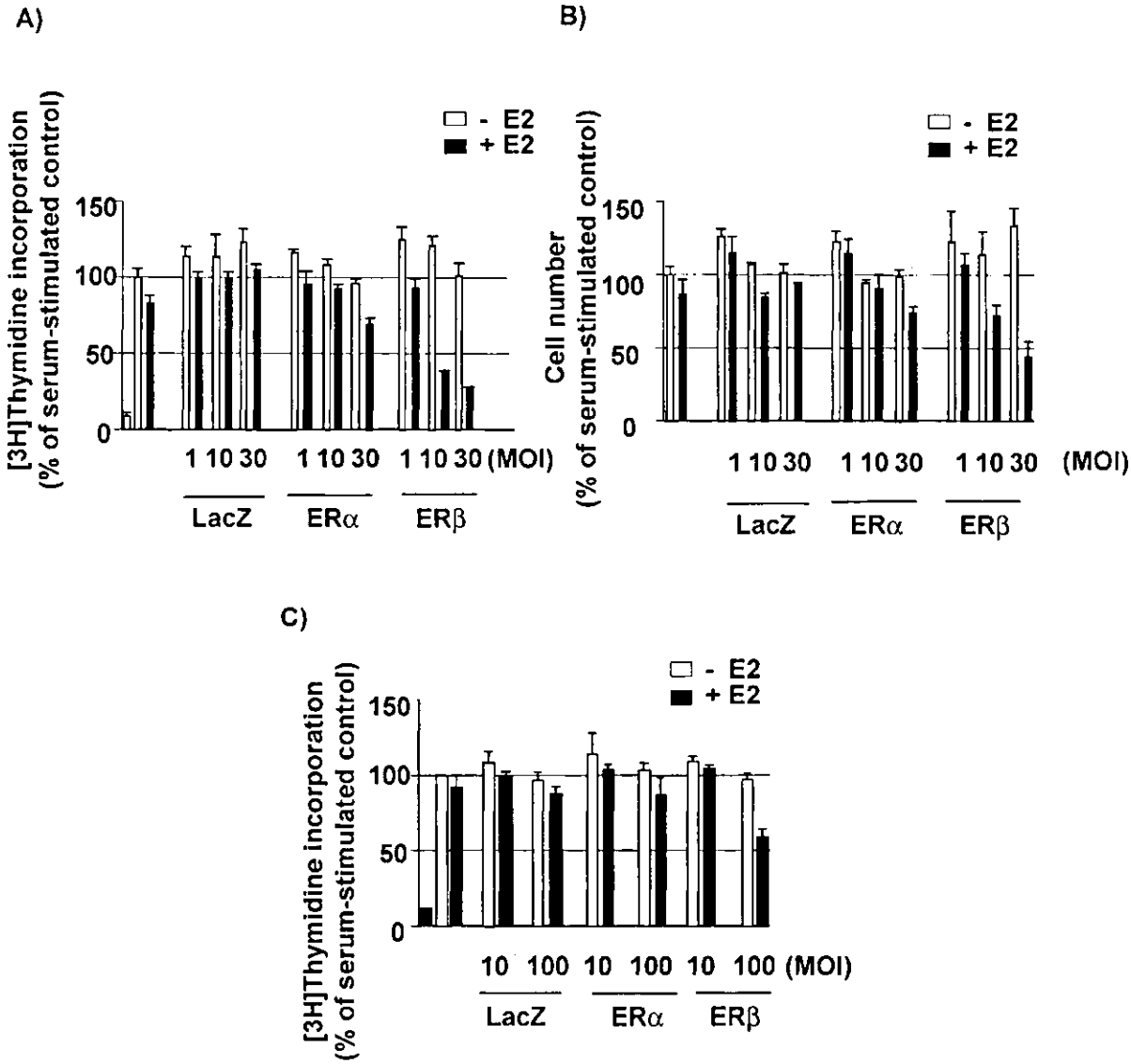


Fig. 2. Inhibition of VSMC growth by adenovirus-mediated transfer of ER gene. (A) Rat VSMCs seeded onto a 24-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (1, 10, and 30 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined after 24 h of stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/l E2, respectively. The three left-sided bars indicate non-infected VSMCs serum free, 5% DCC-FBS in the absence of E2, and 5% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=4). Similar results were obtained in three independent experiments. (B) VSMCs seeded onto a six-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, AxCAERβ, or AxCAERDNβ (1, 10 and 30 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. Cell numbers were counted after 48 h of stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/l E2. The two left-sided bars indicate non-infected VSMCs with 5% DCC-FBS in the absence of E2 and with 5% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=3). Similar results were obtained in three independent experiments. (C) Human aortic VSMCs seeded onto a 24-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (10, and 100 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined after 24 h of stimulation with 20% DCC-FBS in the presence or absence of 100 nmol/l E2, respectively. The three left-sided bars indicate non-infected VSMCs serum free, 20% DCC-FBS in the absence of E2, and 20% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean+S.E. (n=3). *P≤0.05 vs. E2 (−). Similar results were obtained in three independent experiments.

Fig. 1. The endogenous expression of ER subtypes, transcriptional activity of ER, and inhibitory effect of E2 on DNA synthesis in rat VSMCs. (A) RT-PCR was performed using the cDNA of rat ovary as a positive control with (lane 2) or without reverse transcriptase (lane 3) and that of rat VSMCs with (lane 4) or without reverse transcriptase (lanes 5). A single band of predicted size (277 bp for ERα and 278 bp for ERβ, indicated by an arrow) was detected in lane 2 and lane 4. Lane 1 shows the molecular weight marker. (B) VSMCs were transfected with luciferase reporter plasmid containing ERE and pRL-SV40 control plasmid. Twenty-four hours after transfection, the cells were treated with 1–100 nmol/l E2 and/or 10–1000 nmol/l ICI182,780 for 24 h. The values were normalized to the vehicle treatment. Results are shown as mean±S.E. (n=3). *P≤0.01 vs. E2 (−). (C) Serum-starved VSMCs were stimulated with 5% DCC-FBS in the absence or presence of 10–1000 nmol/l 17β-estradiol for 24 h. [^{3}H]Thymidine incorporation into DNA was determined by pulse-labeling for the last 3 h of incubation. Results are shown as mean±S.E. (n=6).

culture dishes [27,28] detected in the luciferase assays, but the activity is not strong enough to influence VSMC proliferation.

3.2. Effect of adenovirus-mediated transfer of the ER subtype gene on growth of VSMCs

To examine the effect of ERα and ERβ gene transfer into VSMCs, we constructed a replication-deficient adenovirus carrying the ER gene, AxCAERα, AxCAERβ, or AxCADNERβ. When AxCALacZ was introduced into VSMCs at more than 30 MOI, DNA synthesis was reduced in a MOI-dependent manner (data not shown). Therefore, we examined DNA synthesis at 30 MOI or less, at which the adenovirus itself did not affect DNA synthesis. When AxCAERβ was introduced into VSMCs, DNA synthesis did not change in the absence of E2. However, in the presence of 100 nmol/l E2, DNA synthesis of VSMCs infected with AxCAERβ decreased strongly compared to that of VSMCs treated with vehicle, in a MOI-dependent manner (Fig. 2A). In contrast, VSMCs infected with AxCAERα at 10 MOI or less did not show an additional reduction in DNA synthesis in the presence of E2, although an inhibitory effect was seen in VSMCs infected with AxCAERα at 30 or higher MOI (Fig. 2A and data not shown). In parallel, the increase in VSMC number stimu-

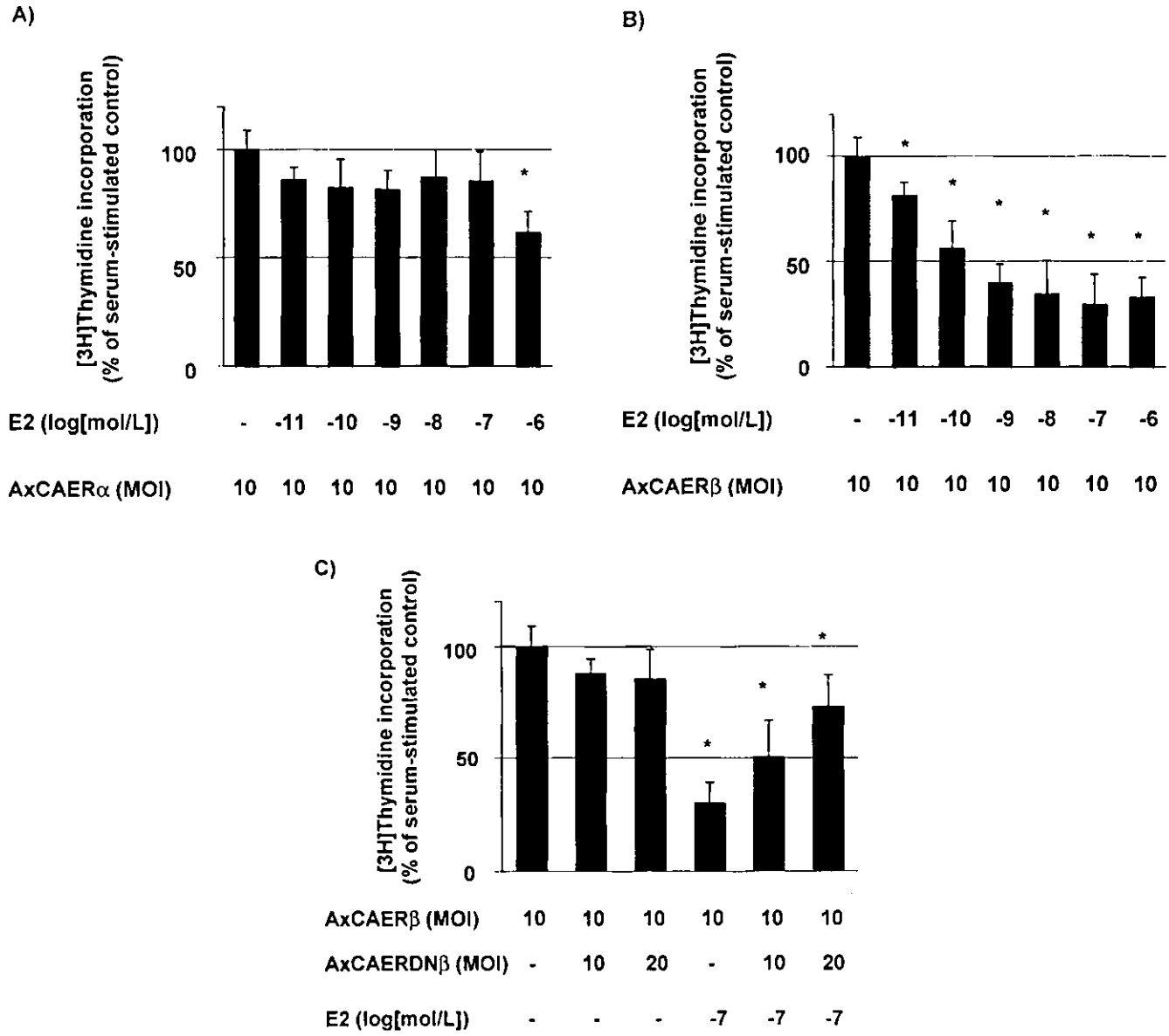


Fig. 3. Dose–response of E2 and receptor dependence of the inhibitory effect of DNA synthesis on adenovirus-mediated transfer of ER genes. VSMCs seeded onto a 24-well plate were exposed to DMEM containing 10 MOI of AxCAERα (A) or AxCAERβ (B) for 2 h, or 10 MOI of AxCAERβ and the indicated MOI of AxCADNERβ (C). After infection, VSMCs were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined 24 h after stimulation with 5% DCC-FBS in the absence or presence of the indicated concentrations of E2. Results are shown as mean±S.E. (n=4).

lated with 5% DCC-FBS for 48 h was attenuated in VSMCs infected with AxCAERβ at 10 and 30 MOI in the presence of E2, but it was not significant in VSMCs infected with AxCALacZ or AxCAERα (Fig. 2B). To exclude the possibility that the findings might be specific for rat VSMCs, we tested human aortic VSMCs and found that the results were comparable in human aortic VSMCs (Fig. 2C). Also, in VSMCs infected with AxCAERβ at 10 MOI, DNA synthesis was significantly inhibited by 0.01–1000 nmol/l E2 in a concentration-dependent manner (Fig. 3B). In contrast, this inhibitory effect was not observed in VSMCs infected with AxCAERα, except in the presence of 1 μmol/l E2 (Fig. 3A). To examine whether the inhibitory effect in VSMCs overexpressing ERβ is actually mediated through ERβ, AxCADNERβ was co-infected with AxCAERβ. The ~70% reduction in DNA synthesis that was observed when AxCAERβ alone was infected was attenuated by co-infection of AxCADNERβ MOI-dependently (Fig. 3C). We also examined the effect of ERα overexpression on ERβ-mediated inhibition of VSMCs. However, AxCAERα at up to 10 MOI did not influence the growth inhibition exerted by AxCAERβ at 10 MOI in the presence of 100 nmol/l E2 (data not shown).

3.3. Production of ER genes and transcriptional activity of ERE in VSMCs infected with ER genes

We examined the mRNA expression of human ERα, ERβ and DNERβ by Northern blot analysis (Fig. 4A and data not shown). Neither ERα nor ERβ mRNA was seen in non-infected VSMCs (data not shown), although both were detected by RT-PCR. Infection of VSMCs with AxCAERα or AxCAERβ induced mRNA expression abundantly in a MOI-dependent manner (Fig. 4A). Similar results were obtained when the membranes were hybridized with the probes for rat ERα and ERβ, indicating that the mRNA expression of endogenous ER was undetectable by Northern blot analysis. Production of the ERα and ERβ protein was confirmed by Western blot analysis (Fig. 4B). The bands corresponding to ERα (65 kD) or ERβ (55 kD) were seen in VSMCs infected with AxCAERα, or VSMCs infected with AxCAERβ, respectively (Fig. 4B) and also in MCF-7 cells which were used as a positive control (data not shown). In parallel with the mRNA expression, the protein expression of the ER subtype was undetectable in non-transfected VSMCs and was increased by overexpression MOI-dependently. We also checked the protein level of both ER subtypes in non-infected cells after the addition of E2. However, E2 did not affect the protein level of either ER subtype under our experimental conditions (data not shown).

To check whether overexpressed ER functions as a transcription factor in VSMCs, the transcriptional activity of ERE was examined (Fig. 5A). VSMCs infected with AxCAERα or AxCAERβ at 10 MOI showed a significant increase in transcriptional activity in the presence of E2 ($P<0.01$ vs. VSMCs infected with 10 MOI AxCALacZ), indicating that both subtypes can work as transcription factors. The results in COS-7 cells (Fig. 5B), in which no endogenous ER is expressed, are clear-cut and suggest that adenovirus infection can induce ERα and ERβ to a similar extent in terms of transcriptional activity. Compared with COS-7 cells, the additional activity caused by ER overexpression was small in VSMCs. The increase, however, was completely abolished by co-infection with AxCADNERβ, suggesting that the transcriptional activity both in non-infected and infected VSMCs in response to E2 was specific for ER.

3.4. Effect of E2 on cyclin A expression

The expression of cyclin A protein in VSMCs was

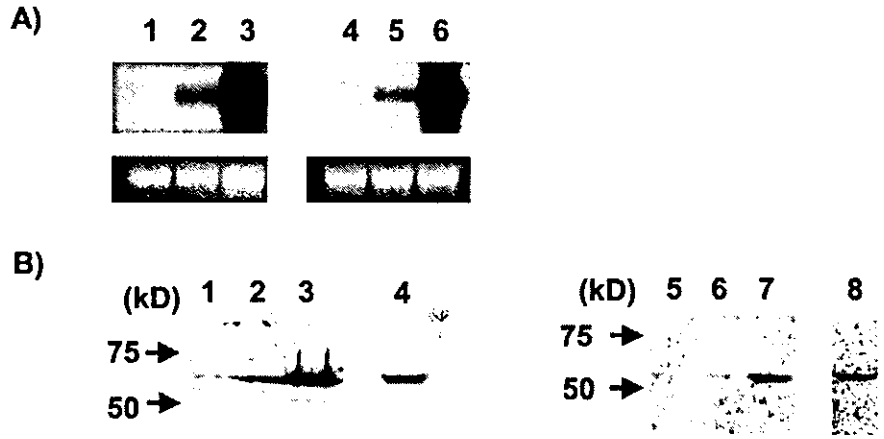


Fig. 4. Induction of ER mRNA and protein in VSMCs by adenovirus-mediated transfer of ER genes. (A) VSMCs were infected without (lanes 1 and 4), or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or AxCAERβ (lanes 5 and 6, respectively). Total RNA was extracted from VSMCs, and Northern blot analysis was performed with 15 μg total RNA per lane. The membrane was hybridized to ^{32}P-labeled ERα or β (upper lane). The 18S RNA is shown as the loading control (lower lane). (B) VSMCs were infected without (lanes 1 and 5) or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or AxCAERβ (lanes 6 and 7, respectively). Positive controls are shown in lane 4 (MCF-7 cells) and lane 8 (rat ovary). Western blot analysis was performed with 40 μg of protein per lane by using an anti-ERα polyclonal antibody or anti-ERβ monoclonal antibody.