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17β-Estradiol inhibits angiotensin II-induced collagen synthesis of cultured rat cardiac fibroblasts via modulating angiotensin II receptors

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Abstract

Circulating endogenous estrogen is considered to be cardiovascular protective, but the underlying mechanisms remain obscure. The cardiac fibroblasts, the most abundant cell type present in the heart, are responsible for the deposition of extracellular matrix. Angiotensin II has been known to stimulate cardiac collagen gene expression. The present study was designed to investigate the effect of 17β-estradiol on the angiotensin II-induced proliferation and collagen synthesis in cultured cardiac fibroblasts by using real-time polymerase chain reaction (PCR), Western blot and 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide proliferation assay. Angiotensin II increased the cell proliferation and synthesis of collagen types I and III. Angiotensin II up-regulated the gene expression of the angiotensin AT_1 receptor and down-regulated the gene expression of the angiotensin AT_2 receptor in cardiac fibroblasts. The effects of angiotensin II was abolished by the angiotensin AT_1 receptor antagonist, losartan, but not by the angiotensin AT_2 receptor antagonist, PD 123319. 17β-Estradiol prevented increases in proliferation and attenuated the collagen synthesis in response to angiotensin II. The increased AT_1 receptor mRNA levels and decreased AT_2 receptor mRNA levels were partially reversed by 17β-estradiol treatment. In conclusion, the down-regulation of angiotensin AT_1 receptor expression and function is likely to be an important mechanism accounting for the inhibitory effect of 17β-estradiol on angiotensin II-stimulated proliferation and collagen synthesis in cardiac fibroblasts. This effect may confer at least in part the cardiac protective action of 17β-estradiol under pathological conditions with increased activity of the rennin–angiotensin system.

Keywords: Cardiac fibroblast; 17β-Estradiol; Angiotensin II; Collagen; (Rat)

1. Introduction

The incidence of cardiovascular diseases in women is lower than age-matched men during their reproductive years (Davidson et al., 2002). Circulating estrogens are suggested to mediate cardiovascular protection in pre-menopausal women (Ho and Mosca, 2002) and estrogen deficiency after menopause is associated with increased cardiovascular risks (Poehlman, 2002). Estrogen replacement therapy delays the onset of cardiac events in post-menopausal women (Hanke et al., 1997; Heckbert et al., 1997). Recent randomized trials of hormone replacement therapy for prevention of heart disease, however, found no overall benefits (Hulley et al., 1998; Rossouw et al., 2002). The exact causes for the different outcomes among these clinical trails are not very unclear.

Cardiac fibroblasts, the most abundant cells present in the heart, are responsible for the main deposition of extracellular matrix. The accumulation of collagen in extracellular space not only leads to cardiac stiffness, but also causes disruption of electrical and mechanical properties of the heart (Weber et al., 1995). Cardiac fibroblasts contain functional estrogen receptors (Mercier et al., 2002) and express angiotensiogen, angiotensin AT_1 and AT_2 receptors (Brilla et al., 1997). Evidence suggests that estrogen interacts with the rennin–angiotensin system (Bohlender et al., 2005; Freshour et al., 2002). Stewart et al. have recently reported that 17β-estradiol inhibits angiotensin II-induced fibroblast-mediated extracellular matrix remodeling by attenuating collagen synthesis (Stewart et al., 2006) and Chao et al. described the inhibitory effect of estrogen on angiotensin II-induced endothelin-1 gene expression in rat cardiac fibroblasts (Chao et al., 2005). Angiotensin II stimulates the collagen

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mRNA expression *in vivo* via angiotensin AT_1 receptors (Scheidegger and Wood, 1997; Yang et al., 1998). The present study aimed to examine the hypothesis that 17β-estradiol reduces angiotensin II-stimulated proliferation and collagen synthesis in cultured cardiac fibroblasts via modulating the expression of angiotensin AT_1 and AT_2 receptors.

2. Methods

2.1. Materials and reagents

Dulbecco's Modified Eagle's Medium (DMEM), 17β-estradiol, ICI 182,780, angiotensin II, PD 123319, methylthiazolyldiphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), collagenase (Sigma-Aldrich Co., St. Louis, USA); goat anti-rat type I and III collagen antibodies, rabbit anti-goat IgG antibody conjugated horseradish peroxidase (Santa Cruz Biotechnology, Inc., CA, UAS); M-MLV reverse transcriptase (Promega Co., WI, USA); SYBR Green QRT-PCR Master Mixture (Toyobo Co., Osaka, Japan); steroid hormones-free fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria); polyvinylidene difluoride (PVDF) membrane (Amersham, NJ, USA); Kodak X-Omat K film (Kodak Co., Xiamen, China); enhanced chemiluminescent-detection kit (ECL-detection kit) (Pierce Biotechnology Inc., IL, USA); RNA extraction Kit (Sangon Co., Shanghai, China); Amicon Ultra 4 (Millipore Co., MA USA). Losartan was a gift from Merck Pharmaceutical Co (USA). Other reagents used in the experiment were of analytical grade.

2.2. Cell culture

Primary cultures of neonatal cardiac fibroblasts were prepared. Briefly, cardiac ventricles from 1- to 2-day-old Sprague–Dawley rats were separated and minced with scissors into small pieces in ice-cold balanced salt solution. Ventricular cardiac fibroblasts were dispersed in 10 ml Spinner solution of the following compositions (in mM): NaCl 116, HEPES 20, NaH_2PO_4 12.5, glucose 5.6, KCl 5.4, and $MgSO_4$ 0.8 (pH 7.35) containing 0.1% collagenase with agitation for 10 min at 37 °C. The digestion steps were repeated five to seven times until the tissues were completely digested. The cells were then combined, centrifuged, and re-suspended in a chilled fetal calf serum.

After final collection, the cells were pelleted by centrifugation (800 ×*g* for 10 min at room temperature). The supernatant was discarded, and the cells were re-suspended in DMEM. The resulting cell mixture was prep-plated for 1 h in a 5% CO_2-containing incubator at 37 °C to plate out cardiac fibroblasts. After removal of the myocyte-enriched medium, DMEM was then added to the pre-plated cardiac fibroblasts which were cultured for 2 days before being passaged. Experiments were performed with cells from passage 3.

2.3. Cell proliferation assay

Cell proliferation was determined with a colorimetric non-radioactive MTT proliferation assay, as described in our previous study (Chen et al., 2005). Cardiac fibroblasts (4×10^5/ml) were seeded in 96-well plates, and treated with or without 17β-estradiol, angiotensin II and ICI 182,780 for 24 h. Thereafter, MTT (5 mg/ml) was added to each well, and plates were incubated at 37 °C for 2 h. The medium was then replaced with 150 μl DMSO and agitated for 10 min. Absorbance at 560 nm was measured using a microplate reader (Packard, Meriden, CT, USA).

2.4. Western blot analysis

Proteins with molecular weights above 30 kD in the cultured medium were separated by Amicon Ultra 4. Immunoblot detections for both type I and type III collagens were performed. In brief, 30 μg of isolated protein was electrophoresed on 8% sodium dodecyl sulphate polyacrylamide gel, and transferred (130 V for 1 h) onto a PVDF membrane. The membranes were treated with blocking solution (TBS pH 7.2, 0.1% Tween, 5% milk) for 1 h, incubated for 12 h at 4 °C with goat anti-rat type I or type III collagen antibodies, diluted 1/1000 in TBS (pH 7.2) 0.1% Tween. After 4 washings for 15 min with TBS (pH 7.2) 0.1% Tween, the membranes were incubated with the second antibody (rabbit anti-goat IgG antibody conjugated horseradish peroxidase, diluted 1/1000 in TBS pH 7.2, 0.1% Tween) for 1 h. They were then washed 4 times for 15 min with TBS (pH 7.2), treated with enhanced chemiluminescent method according to the instruction of ECL-detection kit, exposed to Kodak X-ray film for 0.5–20 min as necessary to detect the signals. The relative intensity of immunoreactive band exposed on the films was quantified by a computer-assisted densitometry program (Smart view, Life Science Research Products and System Engineering).

2.5. RNA isolation

Total RNA was isolated from cultured cardiac fibroblasts according to the protocol of RNA extraction Kits. The amount of RNA isolated was determined by measuring the specific absorbance at 260 nm. The integrity of the RNA isolated was confirmed by agarose gel electrophoresis under denatured conditions. 1 μg of total RNA was used for cDNA synthesis in 20 μl reaction mixture.

Table 1
Primer sets and PCR product characteristics

Target		Oligonucleotide sequence	Tm	Product size (bp)	Genbank correspondence
AT_1	F	5′-CTC AAG CCT GTC TAC GAA AAT GAG-3′	62	204	1270–1473 cDNA (M86912)
	R	5′-TAG ATC CTG AGG CAG GGT GAA T-3′	62		
AT_2	F	5′-ACC TTT TGA ACA TGG TTT G-3′	61	218	2100–2318 cDNA (U01908)
	R	5′-GTT TCT CTG GGT CTG TTT GCT C-3′	60		
GAPDH	F	5′-CCC TTC ATT GAC CTC AAC TAC ATG-3′	60	216	949–1164 cDNA (AF106860d)
	R	5′-CTT CTC CAT GGT GGT GAA GAC-3′	60		

F = forward; R = reverse.

2.6. Primer preparation

For polymerase chain reaction amplification, primers were derived from the Genbank database as described in a previous study (Garcia-Villalba et al., 2003). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene (Table 1).

2.7. Real-time polymerase chain reaction (real-time PCR)

Using Brilliant SYBR Green QRT-PCR Master Mixture, real-time detection of accumulated fluorescence (iCycler iQ® real-time PCR detection system, Bio-Rad, CA) was performed according to the manufacturer's protocol. The PCR primers used in the experiment were cited from previous study (Garcia-Villalba et al.) and synthesizing by PCR procedure was performed with the following time courses: 95 °C for 10 min, 45 cycles at 95 °C for 30 s, 62 °C (for both angiotensin AT_1 and AT_2 receptor) and 60 °C (for GAPDH) for 1 min, and 72 °C for 1 min for amplification. The amplified products were subjected to a stepwise increase in temperature from 55 °C to 95 °C and dissociation curves were constructed.

Target mRNA was quantified by measuring the threshold cycle and reading against a calibration curve. The relative amount of each mRNA was normalized to the housekeeping gene, GAPDH, mRNA. Each sample was tested in triplicate.

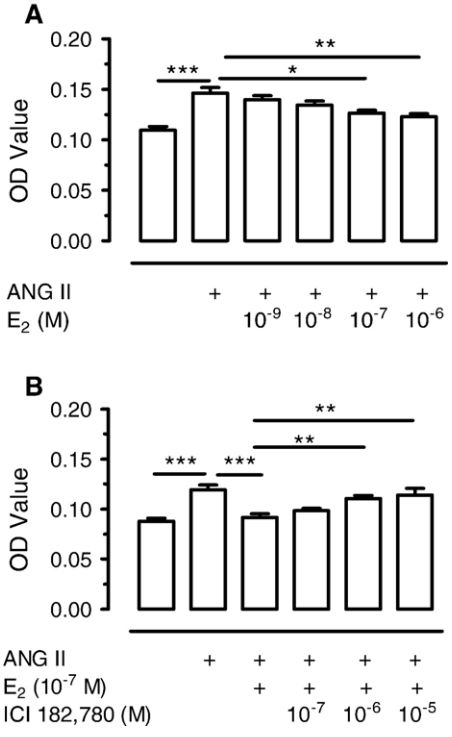

Fig. 1. Analysis of cell proliferation of cultured cardiac fibroblasts by MTT assay. (A) Inhibitory effect of 17β-estradiol (E_2) on 100 nM angiotensin II (ANG II)-induced proliferation. (B) The antagonistic effect of ICI 182,780 (ICI) on 17β-estradiol-induced inhibition of cell proliferation. Data are means±S.D. of 6 separate experiments. Statistical difference between groups is indicated by $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

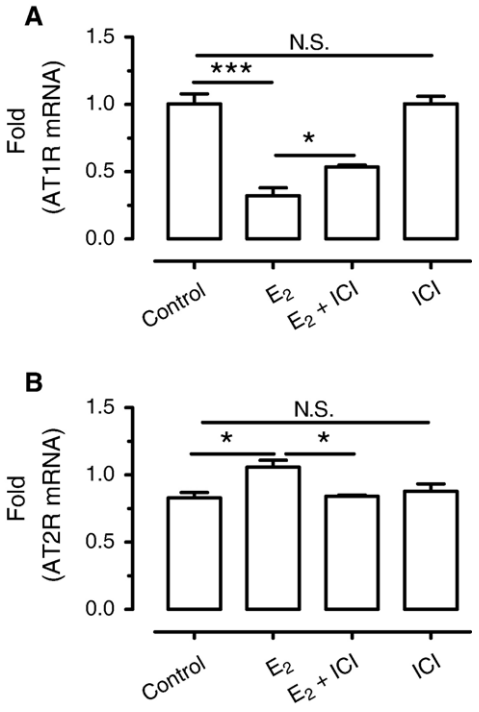

Fig. 2. The effects of 17β-estradiol (100 nM, E_2) and ICI 182,780 (10 μM, ICI) on the mRNA levels of angiotensin AT_1 receptors (A) and of angiotensin AT_2 receptor (B) in cultured cardiac fibroblasts. Data are means±S.D. of 6 separate experiments. Statistical difference between groups is indicated by $^*P<0.05$ or $^{***}P<0.001$.

2.8. Statistical analysis

Data are means ± standard deviation of the mean for the number of experiments indicated. Comparison among different treatment groups was analyzed by one-way analysis of variance followed by Newman–Keul's test. A P value less than 0.05 was considered statistically different.

3. Results

3.1. 17β-Estradiol attenuated angiotensin II-stimulated cell proliferation

Angiotensin II (100 nM, 24 h-treatment) caused a significant increase in the cell number of cultured cardiac fibroblasts. The presence of 17β-estradiol (1 nM–1 μM) inhibited the stimulator effect of angiotensin II on cell proliferation (Fig. 1A) and the 17β-estradiol-mediated effect was largely prevented by ICI 182,780, a classic estrogen receptor antagonist (Fig. 1B).

3.2. Effect of 17β-estradiol on mRNA expression of angiotensin AT_1 and AT_2 receptors

Exposure of cardiac fibroblasts to 17β-estradiol (100 nM) significantly reduced the angiotensin AT_1 receptor mRNA levels and this inhibitory effect was partially antagonized by 10 μM ICI 182,780 (Fig. 2A). In contrast, the angiotensin AT_2 receptor mRNA levels in cardiac fibroblasts were increased by

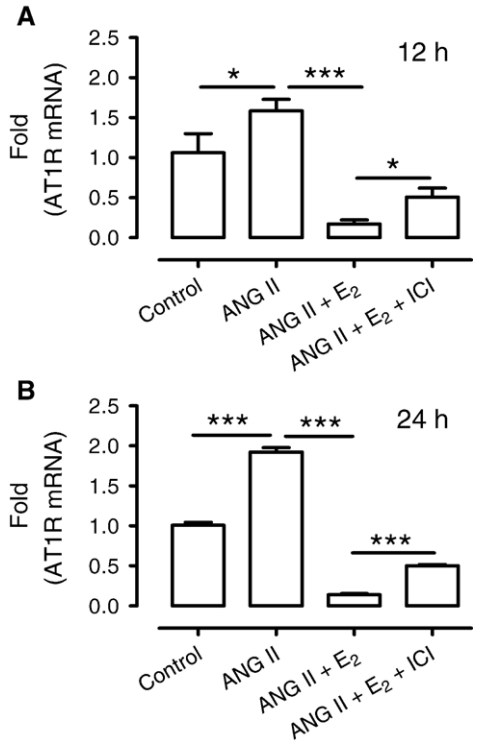

Fig. 3. The effects of 17β-estradiol (100 nM, E_2) and ICI 182,780 (10 μM, ICI) on the mRNA levels of angiotensin AT_1 receptors after 12-hour treatment (A) and after 24-hour treatment (B) with 100 nM angiotensin II (ANG II) in cultured cardiac fibroblasts. Data are means±S.D. of 4 separate experiments. Statistical difference between groups is indicated by (*$P<0.05$ or ***$P<0.001$).

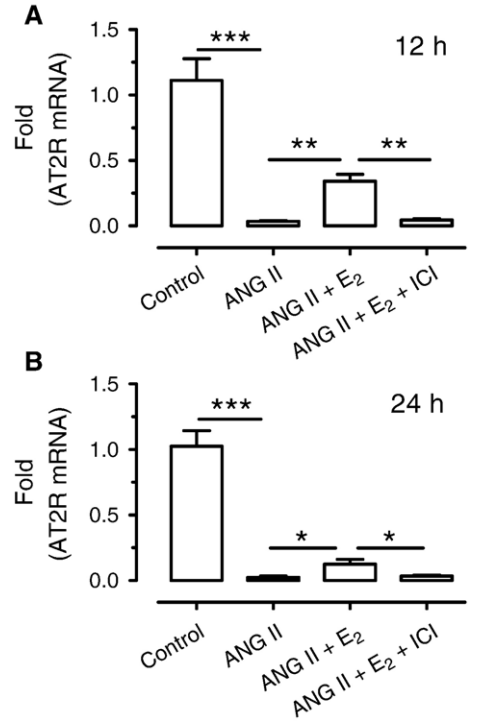

Fig. 4. The effects of 17β-estradiol (100 nM, E_2) and ICI 182,780 (10 μM, ICI) on the mRNA levels of angiotensin AT_2 receptors after 12-hour treatment (A) and after 24-hour treatment (B) with 100 nM angiotensin II (ANG II) in cultured cardiac fibroblasts. Data are means±S.D. of 4 separate experiments. Statistical difference between groups is indicated by *$P<0.05$, **$P<0.01$ or ***$P<0.001$.

17β-estradiol treatment and ICI 128,780 abolished the effect of 17β-estradiol (Fig. 2B). ICI 182,780 alone did not modify the mRNA expression in the presence of angiotensin II (Fig. 2).

Angiotensin II (100 nM, 12 h- or 24 h-treatment) significantly elevated the angiotensin AT_1 receptor mRNA levels in cultured cardiac fibroblasts and this effect was inhibited by 100 nM 17β-estradiol (Fig. 3). The levels of angiotensin AT_1 receptor mRNA in 17β-estradiol-treated cells were even lower than those of untreated cells. ICI 182,780 (10 μM) partially reversed the inhibitory effect of 17β-estradiol (Fig. 3).

Angiotensin II (100 nM, 12 h- or 24 h-treatment)-induced reduction in the angiotensin AT_2 receptor mRNA levels in cultured cardiac fibroblasts was attenuated by 100 nM 17β-estradiol (Fig. 4). ICI 182,780 (10 μM) abolished the effect of 17β-estradiol (Fig. 4).

3.3. Effect of 17β-estradiol on angiotensin II-stimulated collagen synthesis

Exposure of cardiac fibroblasts to 100 nM angiotensin II increased syntheses of both type I (Fig. 5A) and type III (Fig. 5B) collagens. The presence of 100 nM 17β-estradiol reduced

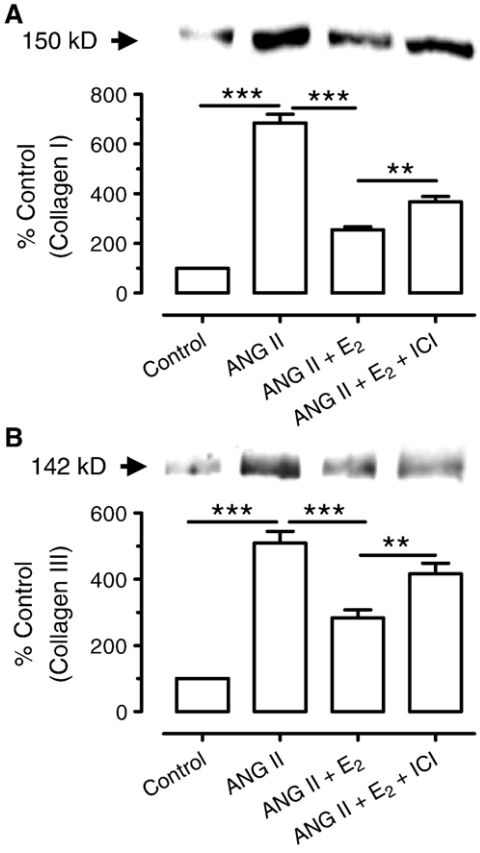

Fig. 5. Changes in the protein levels of collagen I and III (assayed by Western blot method) in the culture medium for cardiac fibroblasts. Angiotensin II (ANG II, 100 nM)-induced increase in the synthesis of collagen I (A) and collagen III (B) was attenuated by treatment with 100 nM 17β-estradiol (E_2). The inhibitory effect of 17β-estradiol was partially antagonized by 10 μM ICI 182,780 (ICI). Data are means±S.D. of 4 separate experiments. Statistical difference between groups is indicated by **$P<0.01$ or ***$P<0.001$.

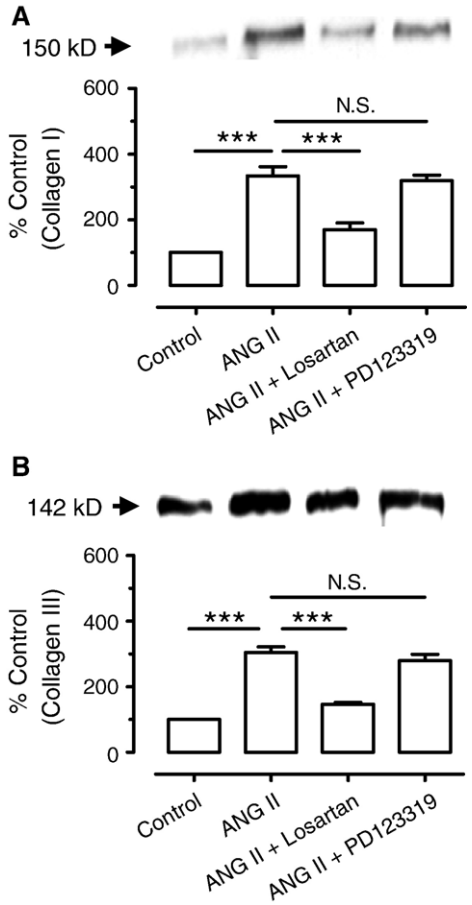

Fig. 6. Angiotensin II (ANG II, 100 nM)-induced increase in the synthesis of collagen I (A) and collagen III (B) was attenuated by treatment with 100 nM losartan but not by 100 nM PD 123319. Data are means±S.D. of 4 separate experiments. Statistical difference between groups is indicated by ***$P<0.001$.

angiotensin II-stimulated collagen synthesis (Fig. 5) and this effect was partially inhibited by 10 μM ICI 182,780 (Fig. 5).

3.4. Effects of losartan and PD 123319 on angiotensin II-stimulated collagen synthesis

Treatment with losartan (100 nM) but not PD 123319 (100 nM) abolished angiotensin II-induced increases in collagen synthesis in cultured cardiac fibroblasts (Fig. 6).

4. Discussion

Although the heart's primary function as a pump depends primarily on the cardiomyocyte, its structural and functional integrity depends largely on the non-myocyte fibroblast (Iwasaki et al., 2005). Cardiac fibroblast is the predominant cell type in the heart, and plays a major role in the deposition of extracellular matrix (Vermeulen, 1995). Collagen type I is the major collagenous product of cardiac fibroblasts, representing 80% of the total newly synthesized collagens. Approximately 20% of the total collagens synthesized are collagen type III while a small proportion is collagen type V (5%) (Iwasaki et al., 2005). In cardiac remodeling, such as hypertrophy and heart failure, excessive deposition of collagens secreted by cardiac fibroblasts may be responsible for the abnormal tissue stiffness and altered cardiac function, and eventually replacing myocytes with fibrotic scars (Brilla et al., 1994; Watanabe et al., 2003).

Activation of the rennin–angiotensin system is a central mediator of progressive cardiac remodeling under abnormal conditions. Angiotensin II, the main effector hormone of the rennin–angiotensin system, modulates cardiac remodeling by causing myocyte hypertrophy and myocardial fibrosis (Wu et al., 2002). Myocardial interstitial changes, characterized by increases in total fibrillar collagens types I and III, and changes in the ratio of type I/III collagens may adversely affect cardiac diastolic and systolic function (Woodiwiss et al., 2001).

Cardiac fibroblasts express both angiotensin AT_1 and AT_2 receptors. Previous evidence suggests that angiotensin II increases the collagen mRNA expression *in vivo* via stimulation of angiotensin AT_1 receptors (Brilla et al., 1997). In contrast, the role of angiotensin AT_2 receptor in cardiovascular physiology is less clear. It has been proposed that angiotensin AT_2 receptor plays a critical role in counterbalancing angiotensin AT_1 receptor-mediated harmful actions to the heart.

Several epidemiological studies suggest that estrogen may be protective against left ventricular hypertrophy, which is an important cardiovascular risk factor for morbidity and mortality. Pre-menopausal women have a lower prevalence of left ventricular hypertrophy than their age-matched male counterparts (Gardin et al., 1995). Left ventricular mass is significantly greater in men than in women. However, conflicting results have been reported concerning the effects of 17β-estradiol on cardiac fibroblast growth. Cardiac fibroblast growth was unaffected by 17β-estradiol (Grohe et al., 1997b), inhibited by 17β-estradiol (Mercier et al., 2002), or even enhanced by 17β-estradiol through a mitogen-activated protein kinase-dependent pathway (Lee and Eghbali-Webb, 1998). Therefore, the exact effect of 17β-estradiol on cardiac fibroblast growth deserves re-examination.

The present study showed that angiotensin II not only stimulated proliferation of cardiac fibroblasts, but also increased the synthesis and secretion of both collagen types I and III. Our results are consistent with those previously reported (Chen et al., 2004). The collagen synthesis-stimulating effect of angiotensin II was mainly mediated by angiotensin AT_1 receptor, but not AT_2 receptor, because this effect was abolished by the angiotensin AT_1 receptor antagonist, losartan. By the contrary, the angiotensin AT_2 receptor antagonist had no effect. This was also in agreement with previously published data *in vivo* (Scheidegger and Wood, 1997; Yang et al., 1998). Treatment with 17β-estradiol attenuated the stimulatory effect of angiotensin II on cell proliferation and collagen synthesis, suggesting that 17β-estradiol could exert a cardiac protective effect by reducing excessive extracellular matrix accumulation and cardiac stiffness due to enhanced activity of the rennin–angiotensin system. Previous reports show contradictory results on the role of 17β-estradiol in the expression of angiotensin receptors. For example, the ratio of angiotensin AT_1/AT_2 receptors was lower in female than in male spontaneously hypertensive rats; treatment with 17β-estradiol decreased the mRNA levels of the angiotensin AT_1 receptor in

kidney and mesenteric vasculature without affecting the mRNA levels of AT_2 receptors (Silva-Antonialli et al., 2004). While, 17β-estradiol was found to up-regulate angiotensin AT_1 and AT_2 receptors in the rat renal tissues (Baiardi et al., 2005). Our study clearly show that 17β-estradiol reduced the mRNA expression of angiotensin AT_1 receptor and prevented angiotensin II-induced increases in angiotensin AT_1 receptor mRNA levels in cultured cardiac fibroblasts. Increases in the angiotensin AT_1 receptor levels may account for angiotensin AT_1-receptor-dependent effect of angiotensin II on cell proliferation and collagen synthesis in cardiac fibroblasts. Thus, angiotensin AT_1 receptor is likely to be an important target for 17β-estradiol-mediated cardiac protection.

Moreover, the inhibitory effect of 17β-estradiol on angiotensin II-stimulated cardiac fibroblasts proliferation and collagen synthesis appears to be mediated by estrogen receptors since ICI 182,780, a classic estrogen receptor antagonist abolished these effects. Both estrogen receptor isoforms (ERα and ERβ) are expressed in cardiac fibroblasts (Grohe et al., 1997a); these nuclear estrogen receptors may mediate a cardiac action of 17β-estradiol (Manabe et al., 2002). However, other studies have described that the anti-proliferative effect of 17β-estradiol is unrelated to estrogen receptor (Dubey et al., 2002; Pasapera Limon et al., 2003). To clarify whether non-genomic mechanism is involved, further investigation is needed.

Another interesting finding of the present study was that treatment with angiotensin II resulted in a significant reduction in the angiotensin AT_2 mRNA levels in cultured cardiac fibroblasts and this reduction was partially prevented by 17β-estradiol. However, the angiotensin AT_2 receptor antagonist did not affect angiotensin II-stimulated collagen synthesis. It is currently unclear about the functional significance of 17β-estradiol in preventing angiotensin II-induced reduction in angiotensin AT_2 receptor mRNA levels. It is possible that maintaining angiotensin AT_2 receptor expression could also contribute to cardiac protection exerted by 17β-estradiol during increased activity of the rennin–angiotensin system.

In summary, angiotensin II increased proliferation and collagen synthesis of cardiac fibroblasts through stimulation of angiotensin AT_1 receptors. Treatment with 17β-estradiol attenuated the stimulatory effect of angiotensin II by down-regulating the expression and/or function of angiotensin AT_1 receptors. This effect may serve an important mechanism by which 17β-estradiol exerts its cardioprotective action even though the role of angiotensin AT_2 receptor in 17β-estradiol-induced cardiac benefits warrants further examination.

Acknowledgments

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