Estrogen modulation of left ventricular remodeling in the aged heart

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Abstract

Objective: To investigate the effects of estrogen on left ventricle (LV) mass and collagen deposition, and on the expression of receptors for estrogen (ERα, ERβ) and Ang II (AT_2R, AT_1R) in the heart of aged female rats. **Methods:** Aged (~12 months old) intact ($n=7$), ovariectomized plus placebo (OVX, $n=7$), and estrogen-replaced (E2, $n=6$) as well as young (~3 months old, $n=4$) female Sprague–Dawley rats were used in this study. After 1 month of treatment, the left ventricular weight/body weight ratio (LVW/BW), changes in myosin heavy chain expression (MHC), matrix metalloproteinase (MMP)-2 activity, the collagen I/III ratio, and the expression of ERs and Ang II receptors in the LV were evaluated. **Results:** In aged rats, OVX increased LVW/BW associated with a higher expression of β-MHC isoform, increased collagen I/III ratio, and decreased MMP-2 activity compared to intact rats. Furthermore, the OVX group had a decrease in ERs α and β as well as AT_2R but an increase in AT_1R expression. Estrogen replacement prevented the effects of ovariectomy on heart remodeling as well as increased further expression of ERβ and decreased AT_1R expression. **Conclusion:** Removal of ovarian hormones increased LV remodeling in the aged rat, which could be attenuated by estrogen replacement. Moreover, regulation of Ang II receptor expression could be a mechanism by which estrogen may modulate heart remodeling.
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1. Introduction

Aging is associated with increased heart remodeling. Senescent hearts present phenotypic changes in myocardial tissue that include hypertrophy and modifications in the contractile machinery of cardiac myocytes, such as a shift of myosin isoforms, as well as alterations on the extracellular matrix (ECM) that lead to collagen accumulation [1,2]. These changes promote fibrosis and ventricular stiffness, which may cause electrical and mechanical alterations; thus, resulting in predisposition to heart failure, arrhythmias, and sudden death [1,2].

Population studies have shown that in women the increment of heart mass for increment of age is higher than in men, with these differences being more apparent after menopause [3,4]. Furthermore, the impact of left ventricular hypertrophy (LVH) on morbidity and mortality is also higher in women [5]. Several studies have also reported that women receiving hormone replacement therapy (HRT) presented lower LV mass and LV dimensions compared with woman without HRT [6,7]. Moreover, HRT has been shown to attenuate the development of LVH in hypertensive postmenopausal women [8].

Although these observations indicate that estrogen may attenuate heart remodeling related to aging, the mechanisms involved in this modulation remain unclear. Estrogen is known to have multiple effects on the heart [9]. Indeed, cardiac myocytes and cardiac fibroblasts contain both known estrogen receptor isoforms (ERα and ERβ) [9]. However, the effect of aging on the expression of ERs in the heart has not been fully addressed.

Angiotensin II (Ang II) is considered to play a key role in the pathophysiology of heart hypertrophy and remodeling [10]. Ang II type I receptor (AT_1) has been shown to be upregulated in senescent hearts as well as in hypertrophied and failing hearts [11]. Estrogen has been

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reported to downregulate the expression of AT_1 [11], reduce the activity of the angiotensin converting enzyme (ACE) [12], and as recently reported, may directly antagonize the growth-induced effects of Ang II by attenuating the AT_1 mediated ERK activation [13]. Furthermore, estrogen downregulates the activity of MAPK pathways, involved in myocyte growth and in the development of heart failure after LVH [14–16]. Although these observations indicate that estrogen can modulate the cardiac effects of Ang II, its effects on the expression of Ang II receptors in the aged heart remain unclear.

In addition to its anti-proliferative effects, estrogen may have antifibrotic effects by both inhibiting collagen synthesis and enhancing its degradation. The effects of estrogen on collagen turnover in the aged heart have also been overlooked. In this study, we evaluated the effects of estrogen on LV mass and collagen deposition in the heart of aged female rats. Furthermore, we investigated the influence of aging and hormone status, on the expression of ERs and Ang II receptors in the heart. We hypothesized that estrogen could attenuate the remodeling changes of the aged heart, and that these changes will be associated with modulation of the expression of Ang II receptors.

2. Methods

2.1. Animal model

Female Sprague–Dawley rats were obtained from Charles River and aged (11–12 months) in facilities at the University of Alberta. Ovariectomies were performed in the aged rats in order to control for variable estrogen levels, which occur as rats approach reproductive senescence. At the time of ovariectomy, rats received either an estrogen pellet (17β-estradiol, 7.5 mg/pellet, 125 μg/day release, Innovative Research of America; $n=6$) or a placebo pellet (Innovative Research of America; $n=7$) subcutaneously. The dose of estrogen took into account the larger size of the aged rats and was calculated based on our previous studies [17], and previous data in the literature [18]. Confirmation of estrogen status was determined by uterine weight. Aged, intact (intact, $n=7$) rats were used as controls for both OVX and estrogen-replaced animals. Young animals ($n=4$) were used as a comparative reference group for the aged rats. After 1 month of treatment, rats were killed on the day of the experiment by exsanguination, under anesthesia from an intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight). The animal protocols were examined and approved by the University of Alberta Animal Welfare Committee which followed the guidelines outlined by the Canada Council on Animal Care.

2.2. Heart preparation

Each heart was rapidly excised and immersed in phos-phate-buffered saline (PBS) and then dissected into left ventricle, left atrium, right ventricle and septum. Sections were weighed and then snap frozen in liquid nitrogen and stored at $-80\,°C$ for subsequent biochemical assays.

2.3. Analysis of myosin heavy chain isoforms

Isoforms of myosin heavy chain (MHC) were separated by SDS–PAGE and quantified by Coomassie blue staining [19]. Myocyte homogenates in $2\times$ Laemmli buffer were incubated at $100\,°C$ for 5 min, loaded onto a 0.75-mm thick 0.1% SDS–4% polyacrylamide gel, and separated (Protean II; Bio-Rad). Gels were fixed, stained with Coomassie blue and dried. Bands representing α- and β-MHC were quantified by densitometric analysis (Quantity One; Bio-Rad).

2.4. Zymography

Homogenized LV tissue was suspended in 2 ml PBS (pH 7.4) with 100 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml leupeptin, and incubated at $4\,°C$ for 20 h. The sample was then centrifuged at $12\,000\times g$ at $4\,°C$ for 10 min. The resulting supernatant was employed for total protein assay and zymographic analysis [20]. To analyze gelatinolytic activity, supernatant were mixed with $6\times$ sample buffer (0.5 M Tris–HCl (pH 6.8), 10% SDS, 50% glycerol, 0.5% bromophenol blue). Twenty-five micrograms of protein was loaded per lane. After electrophoresis, gels were washed 3×20 min in 2.5% Triton X-100 at room temperature, followed by 24 h at $37\,°C$ in enzyme assay buffer (25 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.9% NaCl, 0.05% Na_3N) for development of enzyme activity bands. Thereafter, the gels were stained with 0.05% Coomassie blue, and de-stained in acetic acid and methanol. Zymograms were analyzed for pro- and active-MMP-2 with molecular weights of 72 and 69 kDa, respectively. Gels were scanned using Fluor MultiImager (Bio-Rad).

2.5. Western blot

Aliquots of ground LV were sonicated in homogenization solution (2% SDS, 100 mmol/l dithiothreitol, and 60 mmol/l Tris (pH 6.8) at $4\,°C$. Protein concentrations were determined with the use of the method of Bradford. Four-times SDS gels loading buffer (100 mmol/l Tris–HCl, pH 6.8), 200 mmol/l dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) were added, and samples were heated to $100\,°C$ for 10 min. Twenty-five micrograms of protein of the supernatant was run through a 10% polyacrylamide gel followed by electrotransfer to nitrocellulose at $4\,°C$. Membranes were then incubated in 5% nonfat dry milk in PBS for 3 h and washed with PBS–Tween-(0.1%). Primary antibodies, Collagen type I (1:1000, Santa Cruz), Collagen type III (1:1000, Santa Cruz), Estrogen receptor α (1:1000, Santa Cruz), Estrogen

receptor β (1:500, Santa Cruz), AT_1 (1:500, Santa Cruz), and AT_2 (1:500, Santa Cruz) were incubated with the membrane for 3 h at room temperature. The secondary antibody, goat anti-rabbit antibody or donkey anti-goat (Santa Cruz) was diluted to 1:4000 and incubated with the membrane for 1 h at room temperature. After the last washing step, enzyme-linked chemiluminescence detection was carried out according to the manufacturer's instructions (Amersham).

2.6. Statistical analysis

Data are presented as mean±S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA), and Tukey test or Student–Newman–Keuls for post hoc analysis. Significant differences among groups were defined by a $P<0.05$.

3. Results

3.1. Effect of aging and estrogen on body and uterine weight

Body weight (BW) was significantly increased in aged intact and OVX rats compared to young (Table 1). Estrogen replacement significantly reduced BW compared to the other aged animals. As expected, uterine weights (UW) and the UW/BW ratio, a biological marker of estrogen status, were enhanced in the estrogen group compared to all other groups (Table 1).

3.2. Effect of aging and estrogen on left ventricular remodeling

Left ventricular weight (LVW) was increased in aged, intact rats compared to young, but this can be attributed to the increased body size of the aged animals since LVW/BW were similar (Table 1). Ovariectomy in the aged rats increased LV weight and LV/BW ratio in comparison to intact and estrogen-replaced aged groups (Table 1). Right ventricular weight (RVW) and RVW/BW ratio were not different among groups (data not shown).

Since LV size was different in OVX aged animals, further assessment of remodeling was conducted. In the LV of OVX rats, the expression of α-MHC was reduced which

was prevented by estrogen treatment (Fig. 1). Importantly, there was a shift of MHC with an enhancement of the fetal β isoform in the OVX animals, which was not seen, in the other groups (Fig. 1). In addition, collagen type I/III protein ratio increased 2-fold in OVX rats compared to the other groups (Fig. 2). Moreover, MMP-2 activity was significantly reduced in the OVX group compared to the young or other aged groups (Fig. 3).

3.3. Expression of estrogen and Ang II receptors

Estrogen receptors α and β, and AT_1R, AT_2R expressions were measured in hearts from young, intact, OVX and estrogen groups. ERs were downregulated in the OVX rats, but this was prevented by estrogen treatment (Figs. 4 and 5). Interestingly, ERα but not ERβ was reduced in intact, aged animals compared to the young rats (Figs. 4 and 5). Estrogen replacement enhanced ERα expression to levels similar to that of the young (Fig. 4). For ERβ, estrogen-replacement increased expression to levels higher than either the young or intact, aged rats (Fig. 5), suggesting an enhanced responsiveness of the beta isoform of ER to estrogen.

Ovariectomy enhanced expression of AT_1R that was substantially reduced by the estrogen treatment (Fig. 6). AT_2R expression was reduced in OVX rats that was restored to the levels seen in the young by estrogen replacement (Fig. 7).

4. Discussion

This study evaluated the effects of aging and estrogen on left ventricular weight and remodeling of hearts from aged female rats. Our primary findings are that in the OVX group: (1) LVW/BW was increased, (2) LVH was associated with the expression of the β-MHC isoform, (3) the deposition of collagen I was further increased whereas collagen III was reduced, and (4) MMP-2 activity was also reduced. Estrogen replacement was associated with a decrease in LVW/BW and collagen I/III ratios, reduction in β-MHC expression and enhanced MMP-2 activity in the left ventricle. Furthermore, these changes were associated with increased expression of ERα, β and AT_2R, and decreased AT_1R expression.

These results indicate that in this model of aging, female

Table 1
Body weight, left ventricular weight and uterine weight of animal groups

	Young ($n=4$)	Intact ($n=7$)	OVX ($n=7$)	E2 ($n=6$)
Body weight (g)	280.5±8.0[a]	479.9±13.6[b,c]	497.7±17.2[b]	364.3±19.6[c]
LVW (mg)	603±29[a]	925±47[b]	1228±26[c]	675±33[a]
LVW/BW (mg/g)	2.05±0.04[a]	1.95±0.14[a]	2.49±0.01[b]	1.88±0.12[a]
Uterine W (mg)	538.5±55[a]	809.3±79.3[a]	586.3±62.6[a]	1407.5±87.5[b]
UW/BW (mg/g)	1.91±0.16[a]	1.68±0.16[a]	1.34±0.15[a]	3.92±0.31[b]

Values are mean±S.E.M. Different letters indicate values that are significantly different at $P<0.05$. LVW, left ventricular weight; BW, body weight; UW, uterine weight.
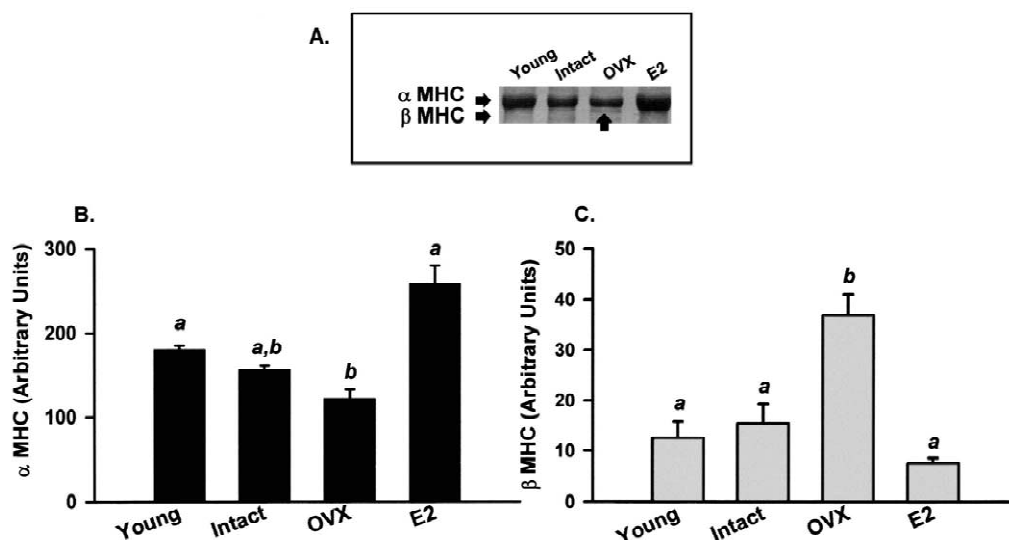
Fig. 1. Estrogen replacement alters α-, β-MHC expression in left ventricle of aged rats. (A) Representative gel for α-, β-MHC expression. In the OVX rats, there was a shift of MHC with an enhancement of the fetal β isoform, which was not seen in the estrogen-replaced group. (B) Summary of α-MHC expression in left ventricles of young (*n*=4) and aged intact (*n*=7), OVX (*n*=7) and estrogen-replaced rats (*n*=6). Bar graphs represent mean±S.E.M. Bars with different letters are significantly different at *P*<0.05.

rats, removal of ovarian hormones enhanced heart re-modeling. These effects could be attenuated by estrogen replacement, suggesting a role of estrogen in the modula-tion of LV remodeling. Moreover, these data also suggest that the local myocardial angiotensin system may be involved in the mediation of estrogen effects on heart remodeling, and that the expression of ERs is modulated by the hormone status.

4.1. Left ventricular mass

Population studies have shown that aging is associated with an increase in LV weight, with a relative higher increase in women than in men [3,4]. Moreover, some studies have shown that blood pressure, body size, and stroke volume may explain only 50% of the variance in mass between men and women [21]. Therefore, other factors such as sex hormones could be important deter-minants of ventricular mass. Indeed, it has been reported that hypertensive premenopausal women have smaller ventricular mass than men matched by age and race, and with the same level of arterial pressure [22]. However, these sex differences tend to disappear in postmenopausal women, suggesting that the ovarian function may protect against LVH and heart remodeling [22].
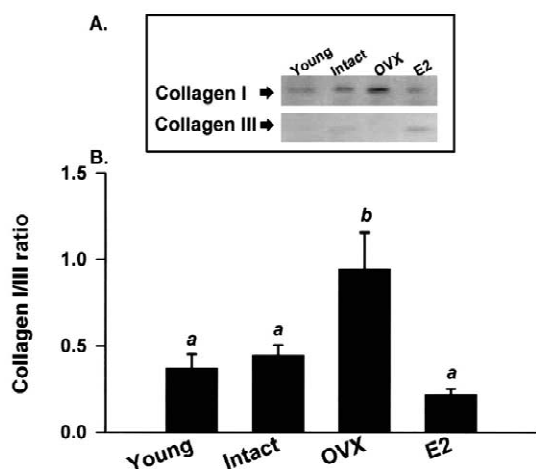

Fig. 2. Effect of estrogen on collagen I/III ratios in aging rat left ventricle. (A) Representative Western blots for collagen I (150 kDa) and III (138 kDa). (B) Bar graphs depict collagen I/III ratios. Results are expressed as mean±S.E.M. Bars with different letters are significantly different at *P*<0.05.
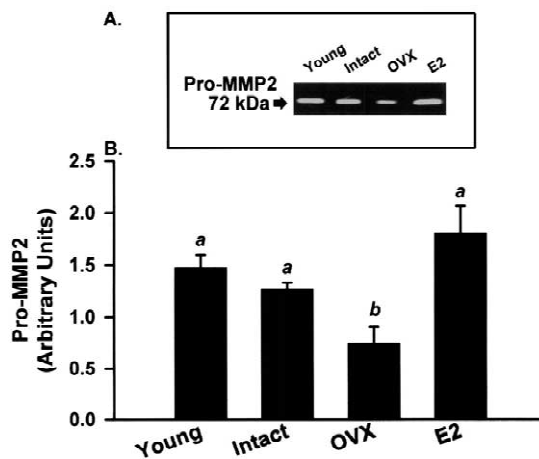

Fig. 3. Effect of estrogen on MMP-2 in aging rat left ventricle. (A) Representative zymography of pro-MMP-2 activity in left ventricles of young (*n*=4) and aged intact (*n*=7), OVX (*n*=7) and estrogen-replaced rats (*n*=6). (B) Summary data for MMP-2 activity. Data are mean±S.E.M. Bars with different letters are significantly different at *P*<0.05.
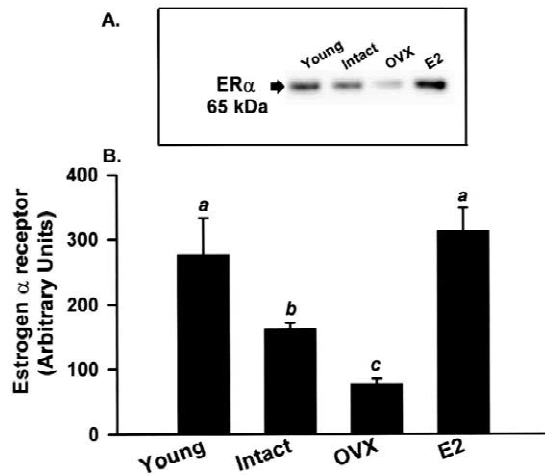
Fig. 4. Estrogen receptor α expression in left ventricle of young ($n=4$) and aged intact ($n=7$), OVX ($n=7$) and estrogen-replaced rats ($n=6$). (A) Representative Western blot for estrogen receptor α (65 kDa). (B) Summary of the densitometric analysis of Western blots. Data are mean±S.E.M. Bars with different letters are significantly different at $P<0.05$.

Fig. 6. Estrogen receptor β expression in left ventricle of young ($n=4$) and aged intact ($n=7$), OVX ($n=7$) and estrogen-replaced rats ($n=6$). (A) Representative Western blot for estrogen receptor β (55 kDa). (B) Summary of the densitometric analysis of Western blots. Data are mean±S.E.M. Bars with different letters are significantly different at $P<0.05$.

In the present study, we found that intact aged rats had an increase in LV weight compared to young rats, but without significant changes in the LVW/BW ratio. However, OVX dramatically increased LVW/BW ratio, and was associated with an enhancement of the expression of the fetal isoform β-MHC, characteristic of hypertrophied and failing hearts, suggesting that estrogen deprivation may greatly affect the heart performance in these aged animals. Accordingly, estrogen replacement significantly reduced the LVW/BW ratio in OVX aged rats. Importantly, the reduction in the LVW/BW ratio was present even with the concomitant decrease in the BW of the estrogen-
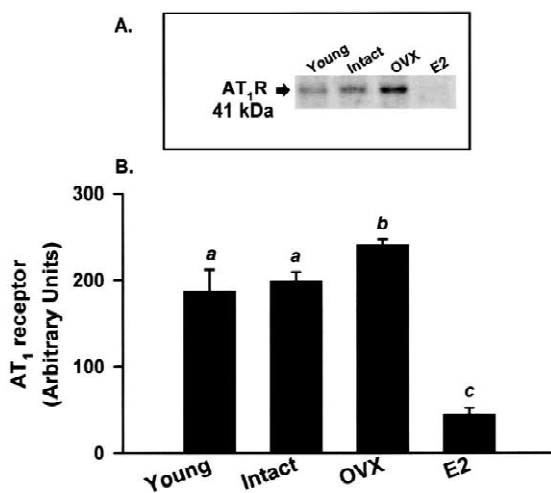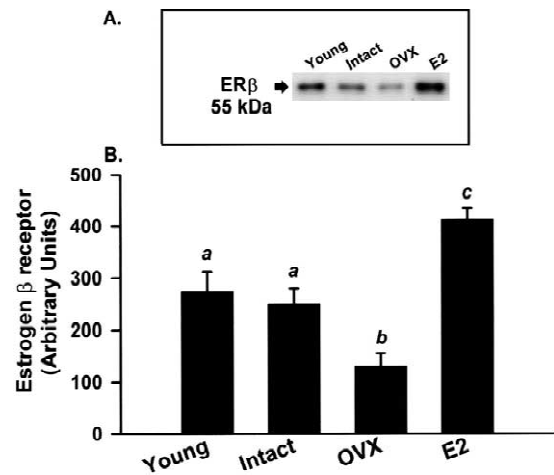
replaced animals. It has been previously reported that estrogen may inhibit the development of LVH in sinoaortic denervated rats [23], and that in ovariectomized mice subjected to pressure overload hypertrophy, estrogen replacement attenuated the hypertrophic response [14]. These observations strongly suggest that estrogen can modulate myocyte hypertrophy.

4.2. Expression of ERs and AngII receptors

It has been described that left ventricles from senescent hearts present an upregulation of the expression of Ang II
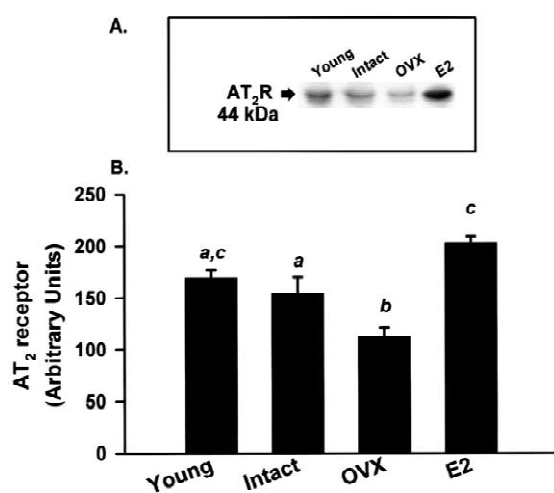

Fig. 5. Angiotensin II type 1 receptor (AT$_1$R) expression in left ventricle of young ($n=4$) and aged intact ($n=7$), OVX ($n=7$) and estrogen-replaced rats ($n=6$). (A) Representative Western blot of AT$_1$R. (B) Summary of the densitometric analysis of Western blots. Data are mean±S.E.M. Bars with different letters are significantly different at $P<0.05$.

Fig. 7. Angiotensin II type 2 receptor (AT$_2$R) expression in left ventricle of young ($n=4$) and aged intact ($n=7$), OVX ($n=7$) and estrogen-replaced rats ($n=6$). (A) Representative Western blots of AT$_2$R. (B) Summary of the densitometric analysis of Western blots. Data are mean±S.E.M. Bars with different letters are significantly different at $P<0.05$.

receptors [24], and of angiotensinogen and ACE myocardial mRNA levels [25]. Interestingly, the upregulation of Ang II receptors does not occur in the right ventricle, where mass does not increase with age [1]. In the present study we found that the LV of OVX rats had an increased expression of AT_1 receptors, which was significantly reduced by estrogen replacement. Indeed, estrogen replacement in the aged animals reduced expression of AT_1 receptors to levels below that of young rats. We speculate that unopposed estrogen in the absence of other ovarian hormones has striking effects on AT_1 receptor expression. Moreover, estrogen replacement was associated with an increase in the expression of AT_2 receptors. Although the role of AT_2 in heart remodeling is not clear, it is thought that AT_2 may counter regulate the signaling of AT_1 [10], and therefore its upregulation could have anti-proliferative effects. Furthermore, AT_2 signaling has been found to have antigrowth effects in many tissues [10]. Thus, estrogen may affect LV hypertrophy by modulating the expression of Ang II receptors on heart tissue. Whether or not estrogen mediates Ang II receptor expression via its own receptor remains to be determined.

It has previously been shown that the two distinct subtypes of estrogen receptor (ERα and ERβ) have been identified in cardiac myocytes and cardiac fibroblasts [9,26]. Our data indicate that there is a down-regulation of both ERα and ERβ in OVX rats, and an upregulation of ERα and ERβ, with estrogen replacement suggesting that estrogen may induce cardiovascular protective effects via mechanisms involving ERs.

4.3. Extracellular matrix of left ventricle

Myocardium contains primarily type I and type III collagen [27]. Their relative concentrations in the heart are important determinants of its mechanical properties [1]. Collagen I has a higher tensile strength compared to collagen III, and its concentration is considered the main contributor to diastolic stiffness [1]. In many species including humans, aging is associated with increased collagen concentration, along with a higher I to III collagen ratio within the heart [27–31]. Moreover, it has been shown that in spontaneously hypertensive rats, hypertrophied ventricles have an increase in the collagen type I to III ratio that can be reversed with ACE inhibitors [32].

In the present study, we found that heart expression of collagen I and III was increased in ovariectomized rats. Moreover, there was an enhancement in the concentration of collagen type I, with a 2-fold increase in the type I to type III collagen ratio that was prevented by estrogen replacement. These findings indicate that estrogen depletion leads to alterations in collagen content in the heart.

Further, it has been described that the activity of MMP-2, which is involved in the breakdown of collagen I, decreases in parallel with age [33]. We found that MMP-2 activity was slightly decreased in LV of aged compared with young rats, but significantly reduced in the ovariectomized group. Moreover, estrogen replacement not only blunted this further reduction in expression, but also was associated with an enhancement of MMP-2 activity to a level similar to that of young rats. These results agree with our previous findings in mesenteric arteries of aged rats, where estrogen replacement was associated with an enhancement of MMP-2 expression [34]. Accordingly, it has been recently reported that estradiol may upregulate the synthesis of MMP-2 in mesangial cells by stimulating the synthesis of the transcription factor AP-2 via the MAPK cascade [35].

It has been recently proposed that an increase of the androgens/estrogens ratio, with a relative predominance of androgens could explain part of the pro-proliferative effects associated with the decreased estrogen levels during menopause [36]. Androgens are known to induce myocyte hypertrophy, and to predispose to collagen accumulation by enhancing the collagen synthesis and its degradation by decreasing MMP-2 activity [37]. Interestingly, some studies have also shown that androgens may increase the expression of AT_1 receptors in some tissues [38]. We speculate that a balance between the effects of androgens and estrogen may modulate heart remodeling, in part through changes in the expression of Ang II receptors.

In summary, our present study is supportive of a role for estrogen in the prevention of LV remodeling associated with aging. Estrogen replacement reduced LV weight and the collagen I/III ratio while enhancing the activity of MMP-2. These effects of estrogen may be mediated through modulation of Ang II receptors, as well as its own receptors.

Acknowledgements

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