Relaxin and Castration in Male Mice Protect from, but Testosterone Exacerbates, Age-Related Cardiac and Renal Fibrosis, Whereas Estrogens Are an Independent Determinant of Organ Size

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This study determined the effects of castration and hormone replacement therapy on the age-related cardiac and renal pathology of male relaxin gene-knockout (RlnKO) and age-matched wild-type (RlnWT) mice and that of aged male aromatase knockout (ArKO) mice, which lack estrogens and have 5–10 times the androgen levels of male wild-type mice. One-month-old RlnWT and RlnKO mice were bilaterally gonadectomized or sham operated and maintained until 12 months. Subgroups of castrated animals received testosterone or 17β-estradiol treatment from 9 to 12 months. Male ArKO mice and aromatase wild-type mice were aged to 12 months. Collected heart and kidney tissues were assessed for changes in organ size and fibrosis. Castration reduced body, heart, left ventricle, and kidney weights in both RlnKO and RlnWT mice, and the cardiac/renal fibrosis that was seen in sham RlnKO animals (all \( P < 0.05 \) vs. respective sham). Testosterone normalized organ weights and organ weight to body weight ratio of castrated animals and increased cardiac/renal collagen concentration to levels measured in or beyond that of sham RlnKO mice (all \( P < 0.05 \) vs. respective castrated mice). Furthermore, expression of TGF-β1, mothers against decapentaplegic homolog 2 (Smad2), and myofibroblast differentiation paralleled the above changes (all \( P < 0.05 \) vs. respective castrated mice), whereas matrix metalloproteinase-13 was decreased in testosterone-treated RlnKO mice. Conversely, 17β-estradiol only restored changes in organ size. Consistent with these findings, intact ArKO mice demonstrated increased cardiac/renal fibrosis in the absence of changes in organ size. These findings suggest that relaxin and castration protect, whereas androgens exacerbate, cardiac and renal fibrosis during ageing, whereas estrogens, in synergy with relaxin, regulates age-related changes in organ size. (Endocrinology 153: 188–199, 2012)

The accumulation of extracellular matrix (fibrosis) is the final common pathway of all chronic cardiac and renal disease. However, despite its pathological significance, progression is variable. For example, although diabetic nephropathy is the single most common cause of renal failure, only about 50% of diabetic patients will develop diabetic nephropathy (1). Although the reason for this is multifactorial, we recognize that there are a number of risk factors that predispose some patients to a higher incidence and a more rapid progression of chronic disease, age (2) and gender (3) being two of the most prominent.

* T.D.H. and C.Z. contributed equally to this manuscript.

Abbreviations: AR, Androgen receptor; ArKO, aromatase knockout; ArWT, aromatase wild-type; BW, body weight; ER, estrogen receptor; ET, 17β-estradiol treatment; LV, left ventricular; MMP, matrix metalloproteinase; PFA, paraformaldehyde; pSmad2, phosphorylation of the TGF-β1 signal transduction molecule, Smad2; RlnKO, relaxin gene knockout; RlnWT, relaxin wild-type; α-SMA, α-smooth muscle actin; Smad2, mothers against decapentaplegic homolog 2; TIMP, tissue inhibitor of metalloproteinase; TT, testosterone treatment; Vg, glomerular volume.
With aging, many subjects exhibit a progressive decline in cardiac and renal function (4). Hemodynamic changes occur in concert with structural changes, including loss of organ mass; hyalinization of arterioles; fibrosis of the tissue parenchyma; and in the kidney, an increase in sclerotic glomeruli. These changes in turn predispose the older heart and kidney to acute organ injury as well as exacerbating progression of chronic organ disease. A growing body of evidence also suggests that the heart and kidneys of elderly people are more sensitive to negative influences from other risk factors such as hypertension, hyperglycemia, hyperlipidemia, and in the context of this study, male gender.

Gender differences in the progression of cardiac and renal disease have been reported clinically (5) and experimentally (6, 7). Females are associated with a lower cardiovascular risk than their male counterparts, but this advantage over male gender disappears after menopause (8). Likewise, several clinical (9) and experimental (3, 10, 11) studies show that the severity of renal diseases are greater in males than females. Our knowledge about how females are protected from the onset of fibrosis is limited but is likely to involve both innate differences in organ size, weight/structure, and hemodynamics as well as the effects of sex hormones (3, 7, 11). Possible explanations include the protective role of estrogens and relaxin or the detrimental effects of androgens.

Estrogens are thought to offer protection in various organ diseases (7, 12). Previous experimental studies have shown that estrogens and their various bioactive metabolites can reduce renal collagen synthesis in vitro (13, 14) and ameliorate damage in some, albeit not all models of cardiac (7, 15, 16) and renal (10, 17) disease. Recent epidemiological studies have shown that both men and postmenopausal women are more prone to chronic cardiac (8) and kidney (18) disease than premenopausal women, again suggesting that estrogens are protective.

The hormone relaxin has long been recognized as a pregnancy-related hormone, based on its ability to remodel the birth canal during parturition via regulation of collagen turnover (19, 20). More recently relaxin has also been demonstrated to have potent matrix remodeling and antifibrotic actions in several nonreproductive organs including the heart and kidney (21–24). The antifibrotic actions of the hormone are mediated via the inhibition of mothers against decapentaplegic homolog 2 (Smad2) phosphorylation (25–27) (a regulatory protein that promotes the activity of the major profibrotic factor, TGF-β1), which in turn disrupts TGF-β1-induced myofibroblast differentiation and collagen production, and also through matrix metalloproteinase-induced collagen degradation (25, 28, 29). Consistent with this, male relaxin gene knockout (RlnKO) mice develop cardiac (30) and renal (31) fibrosis with age, suggesting that relaxin [which is mainly produced in the prostate and/or testes of male mice (19, 20) but is also locally produced within the murine heart and kidney (23)] is also protective. Interestingly, the age-related progression of cardiac and renal fibrosis detected in RlnKO mice were seen only in males, but not females, directly implicating relaxin as a possible factor.

Baylis (32) has shown that both female and ovariectomized female rats are protected from age-related glomerular damage compared with intact males, suggesting that androgens rather than estrogens are the relevant risk factor in disease pathogenesis. Consistent with this factor, castration of a model of fibrotic cardiomyopathy (β2-adrenoceptor transgenic mice) resulted in improved survival rates and significantly ameliorated left ventricular (LV) dysfunction, remodeling, and hypertrophy compared with that measured in intact transgenic mice (7, 33), whereas exogenous testosterone administration has been shown to have adverse effects on collagen deposition in the diseased heart (7, 16) and kidney (9–11).

In this study we examined the interaction of sex hormones (that contribute to the gender based changes in disease pathogenesis) and age on cardiac and renal growth and fibrosis progression. Specifically we determined whether relaxin, 17β-estradiol, and testosterone alter the structure and accumulation of extracellular matrix in the ageing heart and kidney. To do this, we compared various parameters in castrated and hormone-treated male RlnKO and age-matched male wild-type (RlnWT) mice, in addition to that in aged male aromatase knockout (ArKO) mice, which are deficient of estrogens and have 5–10 times higher androgen levels than normal male wild-type (ArWT) mice.

### Materials and Methods

#### Animals

The male RlnWT and gene-knockout (RlnKO) C57B6J × 129SV littermate mice used in this study were generated from heterozygous parents and were individually genotyped and housed in a controlled environment as described previously (30, 31, 34, 35). After weaning (at 3 wk of age), all mice were provided with water and soy-free rodent lab chow (SF00–124; Specialty Feeds, Western Australia, Australia) ad libitum to deprive them of consuming phytoestrogens (which are present in standard rodent lab chow).

The male ArWT and ArKO C57B6J × J129 littermate mice used in this study were also generated from heterozygous parents and were individually genotyped and housed as described before (36, 37). Male ArKO mice are deficient of estrogens and have 5–10 times the androgen levels detected in male ArWT animals (36). As with RlnWT and RlnKO mice, ArWT and ArKO mice...
n = 8–9 per genotype) were maintained on a soy-free diet from the time they were weaned (at 3 wk of age) until they were 12 months old. The experiments detailed below were approved by the Howard Florey Institute’s Animal Ethics Committee, which adheres to the Australian Code of Practice for the care and use of laboratory animals for scientific purposes.

**Gonadectomy and hormone treatments**

To investigate the effects of castration and testosterone treatment (TT) or 17β-estradiol treatment (ET) on age-related phenotypes of adult male mice, 4-wk-old RlnWT mice, and RlnKO mice (n = 16–17 per genotype) were bilaterally gonadectomized as detailed before (38). The mice were then maintained as described above until 12 months of age, a time point at which untreated RlnKO mice develop altered organ size, fibrosis, and dysfunction (30, 31, 34, 35).

To examine the effects of TT and ET in male RlnWT and RlnKO mice, subgroups of castrated animals (n = 5–7 per genotype) were sc administered with either 90-d release pellets of testosterone [18.5 mg; 206 μg/d, a dose that had previously been shown to exacerbate cardiac remodeling and fibrosis (16); Innovative Research of America, Saratoga, FL] or 17β-estradiol [0.25 mg; 2.78 μg/d, a dose that had previously been shown to protect from cardiac remodeling and dysfunction (39); Innovative Research of America], respectively, from 9 to 12 months of age. A separate subgroup of age-matched male RlnWT and RlnKO mice (n = 5–6 per genotype) that underwent sham operations (at 4 wk of age), served as controls.

**Tissue collection**

All sham-operated, castrated, and hormone-treated male RlnWT and RlnKO mice were killed at 12 months of age. Likewise, all male ArWT and ArKO mice were aged and killed when they were 12 months old (for comparison at an equivalent time point). Success of gonadectomy was verified by body and organ atrophy (by weight), whereas the success of TT and ET was confirmed by the reversal of body and organ atrophy. Blood was collected from each animal by cardiac puncture for serum isolation and analysis. The heart and kidneys of all mice were rapidly excised, blotted dry, and weighed. The heart was dissected into LV, right ventricles and atria. LV tissues were further cut into three transverse sections for hydroxyproline analysis, Western blotting, and fixation in 4% paraformaldehyde (PFA) for histology and morphometric analysis. Kidney tissues were cut into four transverse sections (each containing cortex and medulla) for hydroxyproline analysis, Western blotting, and fixation in 4% PFA and methyl Carnoy’s for histochemical analysis. Unfixed tissue portions were snap frozen in liquid nitrogen and stored at −80°C for either hydroxyproline analysis or protein extraction, quantitation, and Western blot analysis. To ensure standardization and to enable intergroup comparisons, each assay used the same portion of LV or kidney tissue from each animal.

**Testosterone RIA**

Serum testosterone levels were measured from castrated and castrated+TT mice in duplicates, using the direct RIA testosterone kit (IMI119; Immunotech, Roissy, France) according to the manufacturer’s instructions. The intraassay variation was 7.9%.

**Assessment of changes in cardiac and renal size**

Body weight (BW), total heart, LV, and kidney weights were measured and expressed as absolute values, whereas heart, LV, and kidney weights were also expressed as respective organ weight to body weight ratios. Additionally, changes in kidney size were assessed by morphometric evaluation of glomerular volume (Vg) and density from PFA-fixed, paraffin-embedded sections, as described previously (12, 40). Vg was calculated as Vg = B/k (Ag)3/2, where Ag is the glomerular cross-sectional area, B = 1.38 is the coefficient for the shape of spheres, and k = 1.1 is the coefficient of size distribution. At least 25 glomerular profiles under a magnification power of ×200 from each sample were quantitated for the number of glomeruli. Three cortical fields under a magnification power of ×10 were used to measure the mean density of glomeruli (number of glomeruli per field), an indirect measurement of tubulointerstitial hypertrophy.

**Table 1.** Body weights, organ weights, and organ wet weights normalized by BW in 12-month-old castrated (Cast.) male wild type (RlnWT) and relaxin gene-knockout (RlnKO) mice, with or without TT or ET

<table>
<thead>
<tr>
<th>RlnWT</th>
<th></th>
<th></th>
<th></th>
<th>RlnKO</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cast.</td>
<td>Cast.+TT</td>
<td>Cast.+ET</td>
<td>Control</td>
<td>Cast.</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td>(n = 6)</td>
<td>(n = 6)</td>
<td>(n = 4)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>36 ± 1</td>
<td>28 ± 1a</td>
<td>33 ± 1b</td>
<td>33 ± 2c</td>
<td>35 ± 2</td>
<td>29 ± 1d</td>
</tr>
<tr>
<td>Heart (mg)</td>
<td>193 ± 5</td>
<td>135 ± 10a</td>
<td>199 ± 11b</td>
<td>234 ± 14d</td>
<td>208 ± 4</td>
<td>147 ± 6a</td>
</tr>
<tr>
<td>LV (mg)</td>
<td>130 ± 2</td>
<td>96 ± 7a</td>
<td>142 ± 6b</td>
<td>168 ± 8c,d</td>
<td>143 ± 4f</td>
<td>105 ± 4a</td>
</tr>
<tr>
<td>Kidney (mg)</td>
<td>229 ± 7</td>
<td>148 ± 8a</td>
<td>284 ± 22b</td>
<td>216 ± 7h,e,a</td>
<td>260 ± 11f</td>
<td>156 ± 10a</td>
</tr>
<tr>
<td>Heart/BW (mg/g)</td>
<td>5.3 ± 0.2</td>
<td>4.9 ± 0.4</td>
<td>6.0 ± 0.2c</td>
<td>7.2 ± 1.0c</td>
<td>6.0 ± 0.4</td>
<td>5.0 ± 0.2d</td>
</tr>
<tr>
<td>LV/BW (mg/g)</td>
<td>3.6 ± 0.1</td>
<td>3.5 ± 0.3</td>
<td>4.3 ± 0.2c,d</td>
<td>5.2 ± 0.6c,d</td>
<td>4.1 ± 0.2f</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Kidney/BW (mg/g)</td>
<td>6.3 ± 0.3</td>
<td>5.4 ± 0.3d</td>
<td>8.5 ± 0.4b,d</td>
<td>6.5 ± 0.1c,e</td>
<td>7.6 ± 0.4f</td>
<td>5.3 ± 0.2d</td>
</tr>
</tbody>
</table>

a P < 0.01 vs. values from genotype-matched control mice.
b P < 0.01 vs. values from genotype-matched Cast. mice.
c P < 0.05 vs. values from genotype-matched Cast. mice.
d P > 0.05 vs. values from genotype-matched control mice.
e P < 0.05 vs. values from genotype-matched Cast.+TT-treated mice.
f P < 0.05 vs. values from RlnWT+/+ control mice.
g P < 0.01 vs. values from genotype-matched Cast.+TT-treated mice.
Histology

PFA-fixed LV tissues from male RlnWT and RlnKO in addition to LV and kidney tissues from male ArWT and ArKO mice were paraffin embedded, cut into 5-μm sections, and stained with picrosorus red to assess the distribution of collagen (red staining), as detailed before (30).

Collagen concentration

Frozen portions of LV or kidney tissue were lyophilized for dry weight measurements before being hydrolyzed in 6 M hydrochloric acid for determination of their hydroxyproline content, as described previously (41, 42). Hydroxyproline values were then converted to collagen content by multiplying by a factor of 6.94 [based on hydroxyproline representing approximately 14.4% of the amino acid composition of collagen in most mammalian tissues (43)] and further expressed as a percentage of the tissue dry weight (collagen concentration).

Western blot analysis

Total protein was extracted from LV and kidney tissues using the method described by Woessner (44) and quantified by the Bradford protein assay (45). To determine the mechanisms involved with hormonal regulation of fibrosis, protein extracts (containing an equal amount of 10–20 μg of total protein/lane) were then electrophoresed under nonreducing conditions on 12.5% acrylamide gels, as detailed before (28). Western blot analyses were performed with a polyclonal antibody to the major profibrotic factor, TGF-β1 (sc-146; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antibodies to phospho-Smad2 [which has been found to be inhibited by relaxin to disrupt TGF-β1 activity (25–27); no. 3108; 1:1000 dilution; Cell Signaling Technology, Danvers, MA], α-smooth muscle actin (α-SMA; a marker of myofibroblast differentiation; M0851; 1:1000 dilution; Dako Corp., Carpinteria, CA), and matrix metalloproteinase (MMP)-13 [a collagen degrading enzyme and rodent ortholog of human MMP-1, which is up-regulated by relaxin (27, 46, 47); IM78T; 1:1000 dilution; Calbiochem, San Diego, CA] in addition to goat antirabbit or antimouse IgG secondary antibodies, respectively. Phospho-Smad2 blots were then stripped and reprobed for unphosphorylated Smad2 (no. 3102; 1:1000 dilution; Cell Signaling Technology).

To determine whether androgen receptor (AR) levels were altered by relaxin, protein extracts from the LV of RlnWT and RlnKO mice (containing an equal amount of 90 μg of total protein per lane) were electrophoresed under nonreducing conditions on 7.5% acrylamide gels. A polyclonal antibody to the AR (sc-816; 1:750 dilution; Santa Cruz Biotechnology) and goat antirabbit IgG secondary antibody were used for analysis.

Densitometry of TGF-β1 dimers (25 kDa), phospho-Smad2 (60 kDa), α-SMA (43 kDa), MMP-13 (55 kDa), and AR was then corrected for corresponding total Smad2 expression.

Reverse zymography

Reverse zymography of LV tissue from 12-month-old intact, castrated, and testosterone- or 17β-estradiol-treated RlnWT and RlnKO mice was used to detect changes in tissue inhibitor of metalloproteinase (TIMP)-1 expression (the natural inhibitor of MMP-13), as described elsewhere (48).

Statistical analysis

Data from male RlnWT and RlnKO mice were analyzed by two-way ANOVA, using the Bonferroni post hoc test to examine the significance of genotype (wild type vs. knockout), treatment (sham, gonadectomy, gonadectomy + hormone treatment), and the interaction between genotype and treatment. Data from male...
ArWT and ArKO mice were analyzed by one-way ANOVA, using the Neuman-Keuls post hoc test to compare data between respective groups. All data are expressed as the mean ± SEM, with P < 0.05 considered statistically significant.

Results

The effects of relaxin, testosterone, and 17β-estradiol on BW and organ weights

The BW, organ weights, and organ weight to body weight ratios of sham-operated, castrated, and TT- or ET-gonadectomized male mice are presented in Table 1. Twelve-month-old sham-operated (control) RlnKO mice had significantly larger LVs and kidneys (by 10 and 14%, respectively) in addition to LV to BW and kidney to BW ratios (by 14 and 21%, respectively), compared with age-matched RlnWT animals (all P < 0.05 vs. 12 month old control RlnWT mice with testes intact). Castration of male RlnWT and RlnKO mice (at 1 month of age) led to a 17–22% reduction in their BW and significantly smaller hearts (by ~30%), LV (by 26–27%), and kidneys (by 35–40%) by 12 months (all P < 0.01 vs. age matched control mice with intact testes). When corrected for BW, the kidney weights of 12-month-old castrated male RlnWT mice and heart and kidneys weights of age-matched castrated RlnKO mice were also smaller than their respective control counterparts (with testes intact).

TT of castrated RlnWT and RlnKO mice normalized the gonadectomy-induced reduction in BW, organ weights, and organ weight to BW ratio to the values seen in respective control animals (all P < 0.05 vs. measurements from respective castrated mice; Table 1). Serum levels of testosterone in castrated + TT mice were 412 ± 172 pg/ml, but were below the detectable level of the testosterone RIA in castrated animals (n = 5 mice/group). The ET of castrated RlnWT mice also normalized the gonadectomy-induced reduction in BW, heart and kidney weights, and heart weight to BW and kidney weight to BW ratios (all P < 0.05 vs. measurements from respective castrated mice) and significantly increased LV weight and LV to BW ratio to levels above that measured in control RlnWT animals (both P < 0.05 control RlnWT mice; Table 1). When administered to castrated RlnKO mice, however, ET did not have any marked effects on BW and only partially normalized absolute heart, LV, and kidney weights, such that the weights of these organs in ET-castrated mice were higher than that measured in castrated mice but lower than that measured in TT-gonadectomized animals (Table 1). When corrected for BW, ET of castrated RlnKO mice appeared to normalize heart, LV, and kidney to BW ratio to that measured in control male RlnKO mice.

The effects of relaxin, testosterone, and 17β-estradiol on mean glomerular volume and density

Despite 12-month-old male RlnKO mice having larger kidney weights and kidney weight to BW ratios compared with age-matched RlnWT animals (Table 1), no significant differences in glomerular volume (Fig. 1, A and B) or density (Fig. 1, D and E) were measured between control male RlnWT and RlnKO mice. Consistent with the smaller kidneys seen in castrated animals, however, both gonadectomized RlnWT and RlnKO mice had smaller glomeruli (Fig. 1, A and B) and an increased glomerular density (Fig. 1, D and E). Conversely, both TT and ET restored glomerular volume (Fig. 1, A and B) and glomerular density (Fig. 1, D and E) in these animals in the
same manner that they restored the castration-induced reduction in kidney weight and kidney weight to BW ratio (Table 1).

Interestingly, no differences in glomerular volume (Fig. 1C) or glomerular density (Fig. 1F) were measured between intact 12-month-old male ArWT and ArKO mice, the latter being estrogen deficient and containing 5–10 times the androgen levels measured in age-matched male ArWT mice.

**The effects of relaxin, testosterone, and 17β-estradiol on collagen concentration and density**

Biochemical analysis of LV and kidney collagen concentration (percentage collagen content to dry weight tissue) was performed by hydroxyproline analysis of equivalent tissue portions, and representative picrosirus-red staining of LV (B) and kidney (C) collagen at 12 months of age are shown. Also shown is the mean ± SE LV (D) and kidney (F) collagen concentration and representative picrosirus-red staining of LV and renal collagen (E) in 12-month-old male ArWT and ArKO mice. Numbers in parentheses represent the number of animals analyzed per group. *, P < 0.01, **, P < 0.01 vs. sham-operated (intact) RlnWT mice (A and C) or age-matched ArWT mice (D and F); #, P < 0.05 vs. sham-operated (intact) RlnKO mice; §, P < 0.05 vs. castrated RlnWT mice; †, P < 0.05 vs. castrated RlnKO mice; ¶, P < 0.05 vs. castration + TT-administered RlnKO mice.
FIG. 3. The effects of sham operation, castration, and TT or ET of castrated RlnWT and RlnKO mice (at 12 months of age) on representative cardiac and renal Smad2 phosphorylation (60 kDa) by Western blotting (A). Shown are composite blots of cardiac and renal pSmad2 levels from sham-operated RlnWT (lanes 1 and 2) and RlnKO (lanes 3–4) mice; castrated RlnWT (lanes 5 and 6) and RlnKO (lanes 7 and 8) mice; and castrated + TT-treated RlnWT (lanes 9 and 10) and RlnKO (lanes 11 and 12) mice in addition to castrated + ET-treated RlnWT (lanes 13 and 14) and RlnKO (lanes 15 and 16) mice (A). Two separate blots of one to two samples per group produced similar results. The pSmad2 blots were stripped and reprobed with unphosphorylated Smad2 (Smad2) for correction. Also shown are the mean ± SE OD and pSmad2, corrected for Smad2 expression, for each of the groups studied (B). Numbers in parentheses represent the number of animals used per group. **, $P < 0.01$ vs. sham-operated (intact) RlnWT mice; ##, $P < 0.01$ vs. sham-operated (intact) RlnKO mice; §§, $P < 0.01$ vs. castrated RlnWT mice; ††, $P < 0.01$ vs. castrated RlnKO mice; +, $P < 0.05$, †, $P < 0.01$ vs. castration + TT-administered RlnWT mice; †††, $P < 0.01$ vs. castration + TT-administered RlnKO mice.

The effects of relaxin, testosterone, and 17β-estradiol on Smad2 phosphorylation

Phosphorylation of the TGF-β1 signal transduction molecule, Smad2 (pSmad2; a recently described target for relaxin activity (25–27)), in the LV and kidney (Fig. 3, A and B) paralleled the changes in collagen concentration and staining (Fig. 2, A–C) that were detected between castrated and TT- or ET-RlnWT and RlnKO mice. Thus, LV and renal expression of pSmad2 was markedly increased in intact 12-month-old male RlnKO mice compared with that measured in age-matched control male RlnWT animals ($P < 0.01$ vs. respective levels in intact RlnWT mice) and completely normalized (both $P < 0.01$) in castrated RlnKO mice compared with that measured in intact male RlnKO animals (Fig. 3, A and B). TT of castrated RlnWT animals again exacerbated LV and renal pSmad2 levels to that measured in intact 12-month-old RlnKO ($P < 0.01$ vs. levels measured in intact and castrated RlnWT mice) and further increased pSmad2 levels in gonadectomized RlnKO to levels above that measured in TT-RlnWT ani-
mice. Cast., Castrated.

The effects of relaxin, testosterone, and 17β-estradiol on TGF-β1 and α-SMA expression

Myocardial expression of the profibrotic stimulator, TGF-β1, and expression of α-SMA (a marker of myofibroblast differentiation) (Fig. 4, A and B) also paralleled the changes in collagen concentration (Fig. 2A), collagen staining (Fig. 2B), and pSmad2 (Fig. 3A) that were detected between castrated and TT- or ET-RlnWT and RlnKO mice. Thus, LV TGF-β1 and α-SMA levels were both significantly up-regulated in intact male RlnKO mice in addition to TT-RlnWT and RlnKO mice (all \( P < 0.01 \) was intact RlnWT and gonadectomized RlnWT and RlnKO mice) but were completely normalized in castrated RlnKO mice and ET-RlnWT and RlnKO animals (Fig. 4, A and B). Furthermore, as with cardiac and renal pSmad2 levels (Fig. 3, A and B), LV TGF-β1 levels were further increased in TT-RlnKO mice compared with that measured in the TT-RlnWT animals (\( P < 0.05 \) vs. levels from TT-RlnWT mice).

**The effects of relaxin, testosterone, and 17β-estradiol on MMP-13 and TIMP-1 expression**

No changes in myocardial MMP-13 (Fig. 5A) or TIMP-1 (Fig. 5B) expression were detected between intact male RlnWT and RlnKO mice compared with levels measured in castrated animals. TT of gonadectomized RlnWT mice induced a trend toward decreased MMP-13 expression and significantly decreased MMP-13 levels when administered to castrated RlnKO mice (\( P < 0.05 \) vs. levels measured in intact and castrated RlnKO mice) (Fig. 5A) without having any effects on TIMP-1 levels (Fig. 5B). In comparison, ET had no marked effects on myocardial MMP-13 (Fig. 5A) or TIMP-1 (Fig. 5B) expression when administered to castrated RlnWT or RlnKO mice.

**The effects of relaxin on AR expression**

To determine whether relaxin had any direct effects on AR protein expression, Western blot analysis of AR levels from the LV of male RlnWT and RlnKO mice was carried out having any effects on TIMP-1 levels (Fig. 5B). In comparison, ET had no marked effects on myocardial MMP-13 (Fig. 5A) or TIMP-1 (Fig. 5B) expression when administered to castrated RlnWT or RlnKO mice.
Densitometry indicated that LV AR expression was higher in RlnKO mice than their WT counterparts (*P* < 0.05), suggesting that relaxin regulates receptor expression (Fig. 6).

**Discussion**

The progression of aging-related pathologies can be influenced by gender in both humans (5, 8, 9) and experimental animals (3, 6, 7, 10, 11). To understand the impact of aging, it is necessary to understand the structural alterations and functional changes in these organs and how they are influenced by other intrinsic risk factors. In this study we therefore examined the interaction of sex hormones, age, and fibrosis progression. Specifically we determined the extent to which relaxin, estrogens, and androgens alter structure and accumulation of extracellular matrix in the aging heart and kidney. Our findings from castrated and hormone replacement-treated senescent male RlnKO mice and male ArKO mice demonstrated that estrogens are an independent determinant of body and organ size but do not play a pivotal role in regulating age-related fibrosis progression; castration protects from, whereas testosterone exacerbates, age-associated cardiac and renal fibrosis; and consistent with our previous findings (30, 31, 34, 35), relaxin protects against age-related fibrosis progression, with the higher levels of androgens in males the most likely cause of the gender-based phenotype in RlnKO mice (30, 31).

In the course of this study, we have also for the first time shown that a deficiency of aromatase results in organ fibrosis and that AR expression is down-regulated by relaxin. Finally, we also now suggest that testosterone and relaxin regulation of TGF-β1 signaling and MMP-13 determine the extent of age-related cardiac/renal scarring and that this may be achieved at the level of AR and/or through the regulation of the TGF-β1/Smad2 axis.

Estrogens have long been considered the main female steroids because of their important roles in the female reproductive system. However the irreversible conversion of androgens to estrogens by aromatase is a final rate-limiting step in sex steroid biosynthesis in both males and females. Therefore, androgens can induce their effects on gene transcription through direct effects on the AR or through their conversion to estrogens, which in turn mediate their effects via the estrogen receptors (ER), ERα and ERβ (49, 50). Although castration of male RlnWT and RlnKO mice resulted in a significant reduction in BW, heart, and LV weight in addition to kidney size (reduced weight, glomerular size, and increased glomerular density) by 12 months of age, this was abrogated by 3 months of TT or ET in RlnWT animals. The fact that both TT and ET prevented these changes suggests that estrogens, converted from androgens, were responsible for the changes in BW and organ size/growth that were measured in normal mice, con-
fibrosis progression in RlnWT and to a greater extent in counterparts, TT but not ET exacerbated LV and kidney fibrosis that was measured in sham-operated RlnKO normalized the age-related increase in cardiac and renal ergistic actions between the two hormones.

mote uterine, cervical, and vaginal weight of ovariectomized and growth via ER, whereas relaxin was shown to promote rat (54) and porcine (55) uterine edema and collagen metabolism in rats (41) and matrix remodeling in gilts (52), pubic symphysial ligament elongation, androgen receptor (AR) protein levels by Western blotting. Shown is a blot of AR expression (110 kDa) from the LV of 12-month-old RlnWT (lanes 1–3) and RlnKO (lanes 4–6) mice. A Smad2 blot was used to demonstrate equivalent loading of protein samples and for correction. Numbers in parentheses represent the number of animals used per group. *, P < 0.05 vs. RlnWT mice.

FIG. 6. The effects of relaxin deficiency on LV AR protein levels by Wester blotting. Shown is a blot of AR expression (110 kDa) from the LV of 12-month-old RlnWT (lanes 1–3) and RlnKO (lanes 4–6) mice. A Smad2 blot was used to demonstrate equivalent loading of protein samples and for correction. Numbers in parentheses represent the number of animals used per group. *, P < 0.05 vs. RlnWT mice.

sistent with its actions in promoting uterine growth (51). This was confirmed in ArKO mice in which the lack of altered organ weights and size was detected (as additionally measured by glomerular density and volume) in animals with 5–10 times the androgen levels of normal male ArWT mice, implying that increased androgen levels alone cannot account for changes in BW and organ size.

Interestingly, ET of castrated RlnKO animals did not fully restore the castration-induced reduction in BW, organ weights, and organ weight to BW ratio to the extent measured in TT-RlnKO mice, further suggesting that relaxin synergistically contributed to the estrogen-induced regulation of cardiac/renal size and growth. These findings are consistent with the synergistic actions of relaxin and estrogens in promoting atrial hypertrophy in ovariectomized female mice (12), mammary gland development in gilts (52), pubic symphysial ligament elongation, and collagen metabolism in rats (41) and matrix remodeling within the endometrial stroma of mice (53). Additionally, separate studies have shown that relaxin and estrogen promote rat (54) and porcine (55) uterine edema and growth via ER, whereas relaxin was shown to promote uterine, cervical, and vaginal weight of ovariectomized, estrogen-primed rats (56), further implicating synergistic actions between the two hormones.

On the other hand, although castration of RlnKO mice normalized the age-related increase in cardiac and renal fibrosis that was measured in sham-operated RlnKO counterparts, TT but not ET exacerbated LV and kidney fibrosis progression in RlnWT and to a greater extent in RlnKO animals, suggesting that androgens are profibrotic through actions via the AR. These findings were supported by the increased cardiac and renal collagen concentration that was measured in 12-month-old male ArKO mice (with increased androgen levels in the absence of estrogens) and are consistent with various studies demonstrating that exogenous testosterone administration adversely affects aberrant collagen deposition in the diseased heart (7, 16) and kidney (3, 11, 57) in addition to those that have shown that androgens rather than estrogens are the relevant risk factor in disease pathogenesis (32). Confirmation of the latter was further supported by the findings that castration of a model of fibrotic cardiomyopathy associated with β2-adrenoreceptor transgenic mice resulted in improved survival rates and significantly ameliorated LV dysfunction, remodeling, and hypertrophy compared with that measured in intact transgenic animals (33).

A surprising, but interesting, finding in this study was that relaxin null mice had greater AR expression than their wild-type counterparts, suggesting a role for relaxin in AR regulation. Enhanced AR expression in relaxin null mice helps explain why the testosterone response in the RlnKO animals seemed to be consistently greater than that seen in RlnWT counterparts. Although beyond the scope of this study, future work could usefully examine the kinetics of this relationship in detail.

Our findings that testosterone exacerbates LV and kidney fibrosis by promoting TGF-β1 expression, Smad2 phosphorylation, and myofibroblast differentiation, although reducing the levels of MMP-13 (which plays a key role in collagen degradation), to a greater extent in the absence of relaxin (in RlnKO mice) in addition to our previously published findings that relaxin protects various organs from age-related fibrosis progression (30, 31, 34, 35), confirms that relaxin and testosterone had opposing effects in regulating aberrant collagen deposition associated with aging. The delineation of the interaction between these two hormones may therefore provide us with a better understanding of differences in pathology observed between males and females. Relaxin, acting through its primary receptor (RXFP1) and the nitric oxide pathway (26), protects the aging and diseased kidney from fibrosis progression by inhibiting Smad2 (a regulatory protein that promotes TGF-β signaling) phosphorylation, and hence the ability of TGF-β1 to promote myofibroblast differentiation in addition to collagen and fibronectin production (25–27). Our current findings further suggest that these markers of matrix synthesis (TGF-β1, pSmad2, α-SMA) and degradation (MMP-13) may represent the level at which testosterone and relaxin regulate the extent of fibrosis progression in males vs. females (which will also depend on the differential expression levels of these hormones be-
tween genders). Interestingly, although a reduction in TIMP-1 activity was involved in the relaxin-mediated protection of diabetic (injury induced) cardiomyopathy in vivo (46), TIMP-1 does not appear to play a significant role and be a target of hormonal regulation in age-related cardiac and renal fibrosis progression. Taken together our findings demonstrate that relaxin opposes the effects of testosterone at both the receptor and TGF-β1 signal transduction level.

In conclusion, the findings of this study have confirmed and provided new insights into the hormonal regulation of BW, cardiac and renal size, and organ fibrosis. The removal of androgens protects the aging heart and kidney from age-related fibrosis progression, whereas the addition of androgens/testosterone exacerbates aberrant matrix accumulation in aging. Estrogens, however, did not appear to play a specific role in regulating age-associated collagen accumulation but were found to be an important determinant of organ size and most likely act in synergy with relaxin to mediate these actions in senescence. Further insight into the opposing actions of relaxin and testosterone in regulating fibrosis progression will improve our understanding of the differences in organ pathogenesis that occur between males and females and our knowledge of the contributions of hormones to gender and aging as risk factors for chronic disease.

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References


37. Hill RA, Pompolo S, Jones ME, Simpson ER, Boon WC 2004 Estrogen deficiency leads to apoptosis in dopaminergic neurons in the medial preoptic area and arcuate nucleus of male mice. Mol Cell Neurosci 27:466–476
42. Samuel CS 2009 Determination of collagen content, concentration, and sub-types in kidney tissue. Methods Mol Biol 466:223–235
47. Bennett RG, Kharbanda KK, Tuma DJ 2003 Inhibition of markers of hepatic stellate cell activation by the hormone relaxin. Biochem Pharmacol 66:867–874
57. Bennett RG, Kharbanda KK, Tuma DJ 2003 Inhibition of markers of hepatic stellate cell activation by the hormone relaxin. Biochem Pharmacol 66:867–874