Aging Reduces the Efficacy of Estrogen Substitution to Attenuate Cardiac Hypertrophy in Female Spontaneously Hypertensive Rats


Abstract—Clinical trials failed to show a beneficial effect of postmenopausal hormone replacement therapy, whereas experimental studies in young animals reported a protective function of estrogen replacement in cardiovascular disease. Because these diverging results could in part be explained by aging effects, we compared the efficacy of estrogen substitution to modulate cardiac hypertrophy and cardiac gene expression among young (age 3 months) and senescent (age 24 months) spontaneously hypertensive rats (SHRs), which were sham operated or ovariectomized and injected with placebo or identical doses of 17β-estradiol (E2; 2 μg/kg body weight per day) for 6 weeks (n = 10/group). Blood pressure was comparable among sham-operated senescent and young SHRs and not altered by ovariectomy or E2 treatment among young or among senescent rats. Estrogen substitution inhibited uterus atrophy and gain of body weight in young and senescent ovariectomized SHRs, but cardiac hypertrophy was attenuated only in young rats. Cardiac estrogen receptor-α expression was lower in intact and in ovariectomized senescent compared with young SHRs and increased with estradiol substitution in aged rats. Plasma estradiol and estrone levels were lower not only in sham-operated but surprisingly also in E2-substituted senescent SHRs and associated with a reduction of hepatic 17β-hydroxysteroid dehydrogenase type 1 enzyme activity, which converts weak (ie, estrone) into potent estrogens, such as E2. Aging attenuates the antihypertrophic effect of estradiol in female SHRs and is associated with profound alterations in cardiac estrogen receptor-α expression and estradiol metabolism. These observations contribute to explain the lower efficiency of estrogen substitution in senescent SHRs. (Hypertension. 2006;48:579-586.)

Key Words: estrogen receptors ■ cardiac hypertrophy ■ aging ■ hypertension

The development of cardiovascular disease in postmenopausal women follows declining estrogen plasma levels with a delay of several years.1 The underlying mechanisms are still largely unknown. Observational studies initially suggested a protective function of postmenopausal hormone replacement therapy (HRT) against heart disease with risk reductions of ≈35% to 50%.2 But controlled clinical end point studies, such as Heart and Estrogen/progestin Replacement Study (HERS) and the recently terminated Women’s Health Initiative (WHI) trial, reported no protective function of HRT consisting of conjugated equine estrogens plus medroxy-progesterone-acetate for the primary or secondary prevention of coronary artery disease.3 In contrast, estrogen supplementation in animal models of human heart disease was mostly associated with beneficial hormone effects. But the majority of these studies were conducted in young animals and, thus, do not take the possibility of aging effects into account, which might, in part, explain controversial results between animal and human studies.4–6 Until recently, these shortcomings could be attributed to the lack of animal models that mimic postmenopausal hypertension. Meanwhile, senescent spontaneously hypertensive rats (SHRs) and aged Dahl salt-sensitive rats have been identified and characterized as suitable models to study postmenopausal hypertension. As reported recently, female SHRs stop cycling at the age of 10 to 12 months and develop further increases in blood pressure thereafter.7 Similar studies have been conducted in Dahl salt-sensitive rats.8 Increased blood pressure in postmenopausal SHRs is associated with the natural cessation of ovarian function and decreasing estrogen plasma levels, which closely resemble observations in postmenopausal women.9 These studies provided substantial and new insight into gender and aging aspects of hypertension. In contrast, aging aspects in the development of cardiac hypertrophy,
gene expression, and function are less well documented, although it seems conceivable that estrogen substitution of young and postmenopausal female SHRs might result in different cardiac phenotypes. Therefore, and because most previous studies including our own were conducted in young animals, we determined cardiovascular function, cardiac gene expression, as well as plasma sex hormone profiles in sham-operated and ovariectomized young and senescent female SHRs on long-term treatment with placebo or identical, body weight–adjusted doses of 17β-estradiol (E2). In particular, we speculated whether the efficacy of estrogen substitution to modulate cardiac hypertrophy and gene expression might be blunted in aged SHRs.

**Methods**

### Animal Model and Treatment

Female SHRs (SHR/Ncr1Ico) obtained from Charles River Laboratories (IFCA/CREDO, Lyon, France) at the age of 12 weeks were kept under standard conditions until reaching postmenopause at 24 months of age (senescent SHRs, n=30). A second batch of animals was purchased from the same supplier at the age of 12 weeks for parallel studies in young SHRs (n=30). Young and senescent SHRs were repartitioned into 6 study groups including sham operation (young: n=10; old: n=10 per group), ovariectomy ([ovx] young: n=10; old: n=10), and ovariectomy plus estradiol supplementation ([ovx+E2] young: n=10; old: n=10). All of the animals were kept under standard conditions including a 12 hours on/off light cycle, commercial diet, and water ad libitum. Ovarectomies and sham operations were performed under isoflurane anesthesia (isoflurane 1.5 volume [vol] percent supplemented by 0.5 L of oxygen per minute) after pretreatment with tribromoethanol/amylene hydrate ([Avertin] 2.5% weight/vol, 6 μL/g body weight IP). Ovariectomized rats were injected either with placebo (95 μL of peanut oil+5 μL of ethanol SC) or a body weight–adjusted dose of E2 (95 μL peanut oil plus 2 μg/kg BW per day of E2 dissolved in 5 μL of EtOH) on a daily basis. All of the animals were treated for 6 weeks before hemodynamic and morphometric measurements. A separate set of young (n=10) and senescent (n=7) SHRs was ovariectomized and treated with E2 under exactly identical conditions to assess hepatic enzyme activity levels of 17β-hydroxysteroid dehydrogenases (17β-HSDs) type 1 (17β-HSD1) and type 2 (17β-HSD2). All of the protocols were reviewed and accepted by the local ethics committee and performed in accordance with the current National Institutes of Health guide for the care and use of laboratory animals.

### Hemodynamic Analysis

Hemodynamic measurements were performed according to published protocols after 6 weeks of continuous treatment under light isoflurane anesthesia and spontaneous respiration (isoflurane 1.5 vol percent supplemented by 0.5 L of oxygen per minute).10 Pressure curves were measured via polyethylene tubing and a microtip manometer (Millar Instruments) calibrated to midchest level. Systolic and diastolic blood pressure measurements were obtained on catheter placement in the thoracic aorta. A calibrated flowmeter (2.5 mm; Statham) was placed around the ascending aorta for continuous measurement of aortic blood flow (cardiac output). Measurements were performed by a trained observer blinded for treatment groups. Animals with nonphysiological heart rate <250 bpm were excluded from hemodynamic analysis (senescent rats: sham: n=3; ovx: n=2; ovx+E2: n=2). Blood pressure in senescent SHRs was also monitored by telemetry (online supplement II, please see http://hyper.ahajournals.org).

### Morphometry

Body weight, heart weight, uterus weight, and tibia length were determined after hemodynamic analysis. Relative heart weight was determined by normalizing absolute heart weight for tibia length.

### Plasma Hormone Level

Plasma samples obtained after hemodynamic analysis and within identical time frames after hormone injection were used for radio-immunoassay (RIA) measurements of E2, estrone (E1), total testosterone, free-testosterone, and 4-androstendione according to the manufacturer’s instructions (Diagnostic Systems Laboratories, Inc). Cross-reactivity of the third-generation estradiol RIA was 6.9% with E1 and with other hormones was not detectable. Cross-reactivity of...
the E1 RIA was 1.25% with estradiol and with other hormones was not detectable. Cross-reactivity for androstenedione RIAs with dihydrotestosterone was 0.08%, with E1 0.03%, and with E2 and testosterone not detectable. Cross-reactivity for testosterone RIAs with dihydrotestosterone was 6.6%, with androstenedione 0.9%, and with E2 not detectable. Information on RIA cross-reactivities were provided by the manufacturer.

Cardiac Protein Expression
Cardiac α and β myosin heavy chain protein (α- and β-MHC) expression was analyzed by SDS-PAGE electrophoresis with subsequent visualization of protein bands by silver staining according to published protocols. Enzymatic activity

Hemodynamic measurements in young and senescent SHRs after sham operation (sham), ovariectomy plus placebo treatment (oxv), and ovariectomy plus substitution with E2 (oxv+E2). HR indicates heart rate; CO, cardiac output; SAP, systolic blood pressure; MAP, mean blood pressure; SV, stroke volume.

*P<0.05 vs ovariectomized young; †P<0.05 vs ovariectomized E2 young.

Statistics
Statistical significance was calculated by 1-way ANOVA followed by Student–Newman–Keuls post hoc testing in all of the experiments except 17β-HSD activities, in which statistical significance was determined by 2-sided t tests. Values are mean ± SEM, and P<0.05 was considered significant.

Results

Morphometry
Body weight was comparable among sham-operated senescent and young rats (Figure 1A). The gain of body weight in ovariectomized animals was attenuated by E2 treatment in young and aged SHRs. Uterus weight, which was not different between sham-operated young and senescent rats, decreased with ovariectomy, but residual uterus weight was higher in senescent SHRs (Figure 1B). Estradiol treatment resulted in increased uterus mass in ovariectomized young and senescent SHRs. Relative heart weight, which was higher in sham-operated senescent compared with young SHRs, increased in young but not in senescent rats on ovariectomy (Figure 1C). Estradiol substitution attenuated cardiac hypertrophy in young but not in senescent rats. Cardiac mass was higher in estrogen-substituted senescent compared with estrogen substituted young SHRs.

Hemodynamics and Telemetric Blood Pressure Analysis
Ovariectomy and E2 substitution did not affect blood pressure among the groups of aged and among the groups of young SHRs (Table). However, mean blood pressure levels were higher in sham-operated and estrogen-substituted senescent compared with young SHRs receiving estrogen substitution. Left ventricular stroke volume and cardiac output were comparable among all of the groups of senescent and young rats. Telemetric blood pressure analysis was in good agreement with invasive measurements and did not reveal a blood pressure-lowering effect of E2 substitution in senescent SHRs (online supplement II).
Plasma Sex Hormone Levels

Plasma E2, E1, total, and free testosterone, as well as androstendione plasma levels, were significantly higher in sham-operated young compared with senescent SHRs (Figure 2). Ovariectomy decreased plasma levels of E2 and E1 in young rats but had no such effect in senescent SHRs. Plasma androgen levels were lower in ovariectomized SHRs of both age groups. Estradiol, as well as E1, levels were higher in young SHRs treated with E2 compared with placebo-treated ovariectomized animals. In contrast, plasma E2 and E1 levels in E2-substituted senescent SHRs were not different from placebo-treated aged rats. E1 and estradiol levels were closely correlated among all of the samples ($P<0.001$; $R^2=0.71$).

Figure 2. Sex hormone plasma levels of young (■) and senescent SHRs (■) after sham operation (sham), ovariectomy plus placebo treatment (ox), and ovariectomy plus substitution with E2 (ox+E2). All of the measurements were performed using plasma samples obtained within identical time frames (15 to 19 hours) after hormone or placebo injection. Bars indicate mean±SEM ($n=10$ animals per group; **$P<0.001$, *$P<0.01$, *$P<0.05$).
17β-HSD1 and 17β-HSD2 Activity
Hepatic 17β-HSD1 activity, which catalyzes the reduction of weak estrogens, such as E1, to potent estrogens, such as E2, was significantly lower in senescent compared with young SHRs receiving estrogen substitution (Figure 5A). Activity levels of 17β-HSD2, which metabolizes E2 to less potent estrogens, such as E1, was comparable among estrogen-treated young and senescent SHRs (Figure 5B).

Discussion
The primary result of this study is the observation that estrogen substitution is significantly less efficient to attenuate cardiac hypertrophy in senescent compared with young female SHRs. Although several biomarkers, such as body weight, uterus weight, and cardiac ERα expression, were responsive to E2 supplementation of senescent SHRs, heart weight and cardiac isomyosin expression were not. Lower E2 plasma levels in sham-operated and in estradiol-substituted senescent rats together with lower 17β-HSD1 activity indicate that aging in SHRs is associated not only with reduced E2 synthesis but also with altered E2 metabolism. These observations could at least in part explain the blunted effect of E2 on cardiac hypertrophy in aged SHRs.

Aging is associated with increased blood pressure and cardiac hypertrophy in postmenopausal women. Blood pressure levels were comparable and not different among all 3 of the groups of young rats and among the different groups of senescent SHRs, which is in line with previous findings, including our own. Telemetric blood pressure measurements in senescent SHRs were in good agreement with invasive blood pressure levels and again did not reveal a blood pressure-lowering effect of estradiol. In line with previous observations, blood pressure differed, however, between some groups of aged and young SHRs, because systolic and mean blood pressure was higher in intact and in estrogen–treated senescent SHRs compared with young rats receiving hormone substitution.

Cardiac hypertrophy, which is frequently associated with hypertension, represents an independent predictor of cardiovascular mortality. Increased cardiac mass in sham-operated senescent compared with young SHRs could result from higher blood pressure levels in aged rats, although statistical significance was not reached in comparisons between treatment groups receiving identical treatment (ie, intact young versus intact and in estrogen–treated senescent SHRs compared with young rats receiving hormone substitution). In line with previous observations, E2 efficiently attenuated cardiac hypertrophy in ovariectomized young SHRs, but blood pressure levels were not significantly different among all of the groups of young SHRs. These findings indicate that estrogens are able to attenuate cardiac growth also by direct and blood pressure–independent mechanisms, such as cardiac ANP expression and mitogen-activated protein kinase activation, as we and others have reported before. But in contrast to young animals, estrogen substitution failed to attenuate cardiac hypertrophy in aged SHRs. These observations are supported by measurements of cardiac isomyosin expression. Cardiac hypertrophy in rodents is associated with a prototypical switch in cardiac isomyosin expression from predominant α-MHC to β-MHC expression. As one would predict from measurements of cardiac mass, estrogen treatment reversed the downregulation of α-MHC in ovariectomized young but not in aged SHRs.
Estrogen effects on cardiac hypertrophy in senescent rats are at present not fully understood. Xu et al.\(^{21}\) reported on increased left ventricular remodeling in ovariectomized adult Sprague–Dawley rats that was prevented by E2 substitution. However, the aim of these studies, which were conducted in normotensive rats, was clearly different from the present study and not designed to compare the efficiency of E2 substitution between young and adult rats.\(^{17}\) The role of estrogens in hypertension and cardiac hypertrophy in aged rats has also been evaluated by Hinojosa-Laborde et al.\(^{8}\) who provided solid evidence for increased blood pressure in ovariectomized compared with sham-operated Dahl salt-sensitive rats that was attenuated by estrogen substitution. But cardiac hypertrophy has so far not been assessed in E2-treated Dahl rats.\(^{22}\) Sharkey et al.\(^{16}\) reported that absolute heart weight was not affected by E2 treatment of aged, heart failure–prone hypertensive rats (SHHF/Mcc-fa\(^{a}\)). However the focus of these studies, which were conducted in intact and heart failure–prone rats, was on young animals. Moreover, the lack of...
complete hemodynamic data, estrogen plasma levels, and estrogen receptor expression did not allow for a more comprehensive analysis and a direct comparison between E2-supplemented young and senescent rats.

General inefficiency of estrogen treatment does not exist in aged rats, because established biomarkers of estrogen activity, such as uterine weight and body weight, responded to hormone treatment in young and aged SHRs as observed here and reported before. Although uterus weight decreased with ovarioectomy in young and in senescent SHRs, the loss of uterine mass was less pronounced in senescent SHRs, which supports the hypothesis that uterus mass in sham-operated aged rats is less representative for estrogen action.

Aging is associated with profound alterations of male and female sex hormone plasma levels in humans and in rodents. Moreover, heart weight and body weight, as well as blood pressure, are subjected to regulation via estrogens and androgens, including their pharmacologically active metabolites. As expected, plasma estradiol levels were lower in ovarioctomized SHRs and restored to physiological levels in E2-treated young SHRs. Lower plasma E2 levels in intact senescent compared with young SHRs match with previous reports and with decreased plasma levels of E1. E1 is generated by different 17β-steroid dehydrogenases that catalyze the conversion of potent into less potent estrogens in an initial step of E2 metabolism. Surprisingly, plasma samples from estrogen-substituted senescent SHRs, which were taken within identical time frames after hormone injection in young and aged animals, revealed low E2 levels that were indistinguishable from ovarioctomized and placebo-treated senescent SHRs. These results, which are supported also by low E1 plasma levels in E2-substituted senescent SHRs, suggest that estrogen metabolism differs between young and senescent SHRs. Interestingly, E2 plasma levels were also lower in aged compared with young Dahl salt-sensitive rats receiving E2 supplementation via silastic implants (30±2 versus 20±3 ng/mL; young versus old; P<0.05). But these findings in 12-month compared with 4-month-old rats were not explicitly interpreted as an indicator of increased E2 metabolism. The present study, thus, substantiates previous findings and provides completely independent evidence for aging-associated alteration in estrogen metabolism.

Estrogen metabolism is initiated by 17β-HSDs, which are expressed in multiple isoforms in multiple tissues and catalyze not only the conversion of potent into less potent estrogens but also opposite reactions yielding high affinity from low-affinity ER ligands. Oxidative 17β-HSDs, which include isoforms 2, 4, 8, 10, and 11, convert high-affinity to low-affinity estrogen receptor ligands. Comparable hepatic 17β-HSD2 activity levels among E2-substituted young and senescent SHRs suggest that 17β-HSD2 is most likely not responsible for lower estradiol plasma levels in estradiol-substituted senescent SHRs. However, we cannot rule out that oxidative 17β-HSD isoforms other than 17β-HSD2 might show increased activities in senescent SHRs. In contrast to oxidative enzymes, reductive 17β-HSDs, such as the type 1 enzyme, catalyze the synthesis of potent, high-affinity estrogen receptor ligands, such as E2, from low-affinity precursors, such as E1. E1 plasma levels in senescent SHRs provide a suitable substrate pool for 17β-HSD1 to catalyze the formation of E2, and it is therefore conceivable that lower hepatic 17β-HSD1 activities in senescent compared with young SHRs might have contributed to lower E2 plasma levels. Although the extent to which 17β-HSD1 regulates estradiol plasma levels has up to now not been determined, different hepatic 17β-HSD1 activity profiles in young and senescent SHRs further support the idea that aging is associated with functionally important alterations in estrogen metabolism.

The biological function of estrogens is mediated by 2 different estrogen receptor subtypes, ERα and estrogen receptor β (ERβ), which are functionally expressed in numerous tissues and cell types, including cardiac myocytes and vascular cells. In contrast to previous studies, which failed to detect measurable amounts of ERα and ERβ protein in the mouse heart, several independent studies reported on robust cardiac expression levels of both ER subtypes in rats and humans. However, cardiac expression levels of ERα and ERβ may not only vary between different species but also among young and senescent SHRs. Therefore, it is interesting to note that cardiac ERα expression was significantly lower in sham-operated and ovarioctomized aged rats, whereas ERβ was detected at comparable amounts in the heart of young and senescent rats. The observation that estradiol upregulates ERα expression in vascular cells suggests that lower plasma E2 levels might result in a downregulation of cardiac ERα expression. This hypothesis is supported by increased cardiac ERα content in E2 supplemented senescent SHRs; although plasma E2 levels remained low in these rats, ERα-mediated signal transduction seems, at least in part, to be functional in aged rats.

Perspectives
The current study provides first evidence for lower efficacy of estrogen substitution and altered E2 metabolism in senescent SHRs. Estrogen substitution in postmenopausal women is frequently initiated during the transition phase to menopause. Because SHRs stop cycling at ~12 months of age, estrogen substitution in ovarioctomized 24-month-old senescent SHRs does not resemble estrogen replacement at an early postmenopausal stage. Further studies will, thus, be required to determine whether initiation of estrogen replacement in SHRs during the decline of endogenous estrogen plasma levels is more efficacious than in senescent SHRs. Further studies will also be required to determine the functional relevance of individual 17β-HSD isoforms that have not yet been studied in young and in senescent SHRs.

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References

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