Estrogen, Progesterone, and Aldosterone Interactions

Medroxyprogesterone Acetate But Not Drospirenone Ablates the Protective Function of 17β-Estradiol in Aldosterone Salt–Treated Rats


Abstract—Controversial results obtained from human and animal studies on the prevention of heart disease by estrogens and progestins warrant a better understanding of nuclear hormone receptor function and interaction. To address this issue and taking into account that effects of synthetic progestins are not only referable to action through the progesterone receptor but may also be mediated by other steroid receptors, we characterized cardiovascular function and inflammatory gene expression in aldosterone salt–treated rats on long-term administration of 17β-estradiol, medroxyprogesterone acetate, and drospirenone, a new progestogen exhibiting antimineralocorticoid activity. The complex pattern of cardiovascular injury in ovariectomized Wistar rats induced by chronic aldosterone infusion plus a high-salt diet was significantly attenuated in sham-ovariectomized rats and by coadministration of 17β-estradiol in ovariectomized animals after 8 weeks of continuous treatment. The beneficial role of 17β-estradiol on blood pressure, cardiac hypertrophy, vascular osteopontin expression, perivascular fibrosis, and impaired NO-dependent relaxation of isolated aortic rings was completely abrogated by coadministration of medroxyprogesterone acetate. In contrast, drospirenone was either neutral or additive to 17β-estradiol in protecting against aldosterone salt–induced cardiovascular injury and inflammation. The current results support the hypothesis of complex interactions among estrogen, progesterone, glucocorticoid, androgen, and mineralocorticoid receptor signaling in cardiovascular injury and inflammation. Novel progestins, such as drospirenone, confer superior effects compared with medroxyprogesterone acetate in a model of aldosterone-induced heart disease because of its antimineralocorticoid properties. (Hypertension. 2006;48:994-1001.)

Key Words: aldosterone ■ estrogen ■ medroxyprogesterone-acetate ■ cardiac hypertrophy

Experimental studies provide ample and solid evidence for a protective effect of unopposed estrogens in animal models of human heart disease.1 These reports are in contrast with the neutral or negative outcome of clinical trials on hormone replacement therapy, which consists of combined estrogen and progestin treatment, in the prevention of coronary artery disease in postmenopausal women.2,3 In experimental models, estrogens regulate cardiac and vascular gene expression and function via 2 distinct nuclear estrogen receptors, estrogen receptor-α and -β (ERα and ERβ), which are coexpressed and may functionally interact with the mineralocorticoid receptor (MR), the androgen receptor, and the progesterone receptor (PR) in vascular smooth muscle cells, fibroblasts, endothelial cells, and cardiac myocytes.4,5

In contrast to estrogens, which are highly selective ligands for ERα and/or ERβ, progestogen effects are not only mediated by PRs, but most synthetic progestins also interact with other steroid receptors, such as the androgen, the glucocorticoid, and the MR.6 Progestins might block not only estrogen activity in reproductive tract organs but also in cardiac and vascular cells.7,8 Accordingly, several authors have speculated that such interactions may be responsible for the lack of protective effects in the Women’s Health Initiative Trial.4 Of note, medroxyprogesterone acetate (MPA), in addition to its progestogenic activity, also exhibits androgenic and glucocorticoid activity.9,10 But the role of progestins in cardiovascular disease remains poorly defined, although synthetic progestogens, including MPA, may have played a relevant role in important clinical trials on hormone replacement therapy in cardiovascular disease. Drospirenone, which is a new progestin derived from 17α-spirolactone, closely matches the pharmacological profile of natural progesterone but is devoid of androgenic or glucocorticoid activity and has a higher antimineralocorticoid potency.11-13

Mineralocorticoids play an important physiological role in fluid and electrolyte balance. However, disproportionate elevation of aldosterone serum levels results not only in severe
vascular inflammation, fibrosis, hypertension, and cardiac myocyte hypertrophy in rodents but may also play a causative role in ≤10% of patients diagnosed previously with essential hypertension.14-16 Increased aldosterone serum levels have also been shown to promote the development of congestive heart failure, and MR antagonists, such as spironolactone or eplerenone, improve clinical symptoms and prolong survival in heart failure patients.17-19

Classical hormone replacement therapy may, thus, interfere with mineralocorticoid functions. Accordingly, interactions among nuclear hormone receptors for estrogens, progestins, and mineralocorticoids are of specific interest in cardiovascular disease. To address the issue, we studied the effect of 17β-estradiol and synthetic progestins on cardiovascular function, inflammatory injury, and gene expression in aldosterone salt–treated rats as an established model of disproportionate aldosterone activity.15 Specifically, we hypothesized1 that 17β-estradiol would attenuate the detrimental effects of excessive MR activation,2 that MPA would antagonize the protective function of estrogen,3 that MPA would aggravate MR-mediated cardiovascular injury, and4 that drosperine, as a progestin with antimineralocorticoid activity, might confer more favorable effects than MPA on cardiovascular function and inflammatory gene expression.

Methods

Animal Model and Treatment

Female Wistar rats were obtained from IFFA CREDO (Lyon, France) at the age of 12 weeks. Animals in group 1 were sham operated; animals in group 2 were ovariec-tomized (ovx) and uni-nephrectomized (npx) by right-sided nephrectomy. Groups 3 to 10 were subjected to ovariectomy and uninephrectomy plus aldosterone-salt treatment (AST rats; 0.75 µg aldosterone per hour via Alzet pumps plus 1% NaCl added to tap). AST rats were treated as follows: group 3: placebo; group 4: 17β-estradiol (2 µg/kg per day); group 5: 17β-estradiol (E2) plus spironolactone (20 mg/kg per day); group 6: E2 plus drosperine low dose (3 mg/kg per day); group 7: E2 plus drosperine medium dose (9 mg/kg per day); group 8: E2 plus drosperine high dose (30 mg/kg per day); group 9: E2 plus MPA (3 mg/kg per day); and group 10: placebo plus MPA. Animals in group 11 were sham-ovx, uni-npx, and received AST treatment as described before. Steroids were dissolved in ethanol and adminis-tered by daily subcutaneous injections using either peanut oil (17β-estradiol) or castor oil (MPA, drosperine) as a carrier; spironolactone was supplied with a tap.7,13,20 All of the surgical procedures were carried out under isoflurane anesthesia (isoflurane 1.5 vol% supplemented by 0.5 L of oxygen per minute) after pretreatment with tribromethanol/amylene hydrate (Avertin; 2.5% 1.5 vol% supplemented by 0.5 L of oxygen per minute).21 Left ventricular pressure curves were recorded from the left ventricular cavity, and systolic and diastolic blood pressure measurements were obtained on catheter placement in the thoracic aorta. Measurements were per-formed by a single, trained observer blinded for treatment groups.

Morphometric Analysis

Body weight, heart weight, uterus weight, and tibia length were measured after hemodynamic analysis. Relative heart weight was calculated from absolute heart weight and tibia length to avoid a bias of the results by the adipose phenotype ovx rats. Serum E2 and angiotensin II (Ang II) levels were measured by radioimmunoassays (E2: DPC-Biermann; Ang II: Peninsula). Hearts were dissected into apex, midventricle, and base. The midventricular section was em-bedded into TissueTec OCT (Sakura) and cut into 5-µm sections for hematoxylin and 6-µm sections for picro sirius red staining. Cardiac myocyte cross-sectional areas were calculated from manual tracings of 80 randomly selected cardiac myocytes from each animal (total measurements: 7,900 myocytes) using the “Image J” software (NIH).22 Perivascular collagen accumulation was quantified around 6 randomly selected coronary arteries in 3 nonadjacent sections from each animal (total measurements: 1,800 vessels) using the image analysis system Scion (NIH).23 Media and intima areas and intima :media ratios were measured in 3 nonadjacent aortic sections from each animal (total measurements: 295 sections) by manual tracing of the intima–media border and media–externa border using the Image J software. Ueri were fixed in buffered formaldehyde, cut into 4-µm transverse sections, and stained with hematoxylin/eosin for quantitative evaluation of luminal epithelial cell height using an Axioskop 2 microscope and a KS 400 imaging system (Carl Zeiss Vision GmbH).

Vascular Reactivity Studies

Tissue harvest and organ chamber experiments were performed accord-ing to published protocols.24 The aorta was isolated after hemodynamic analysis in a no-touch technique and placed into modified Krebs–Ringer bicarbonate solution (4°C). Aortic rings were mounted on tungsten stir-ups, placed in an organ bath containing 10 mL of modified Krebs–Ringer bicarbonate solution (37°C [pH 7.4] and 95% O2/5% CO2), connected to force transducers (Föhr Medical Instruments), equilibrated, and stretched to optimum passive tension (2.5-2.0 g). Rings were preconstricted with phenylephrine to ~70% of 100 mmol/L KCl-induced tension and relaxations to acetylcholine ([ACH] 10-10-5 mol/L) or sodium nitroprusside ([SNP] 10-11-10-5 mol/L) were obtained.

Immunohistochemistry

Aortic sections (2 mm) were mounted on glass slides, fixed with 4% formaldehyde in 100 mmol/L of PBS (pH 7.4) before O/N incubation at 4°C with primary antibodies directed against osteopontin (Abcam rabbit polyclonal, 1:100).25 Osteopontin expression was visualized using a commercial kit (Vectastain ABC Kit diaminobenzidine, anti-rabbit IgG, Vector Labs) and quantified using the Scion soft-ware.26 Three randomly selected fields from 3 nonadjacent sections per specimen were analyzed among 4 randomly selected animals per group (total measurements: 1,100 fields). Immediately adjacent sections, in which osteopontin antibodies were omitted, served as negative controls. Nuclei were stained with Hematoxylin QS (Vector Labs).

Statistics

Statistical significance was calculated by 1-way ANOVA followed by Student–Newman–Keuls post hoc testing. Values are mean±SEM, and P values <0.05 were considered significant.

Results

Global and Hemodynamic Measurements

Relative heart weight was significantly higher in estrogen-depleted AST rats compared with sham-operated or ovx and npx animals (27.3±3.5% versus sham; P<0.01; Table). Cardiac mass was lower in AST intact and in ovx AST rats receiving 17β-estradiol substitution (14±2.4% versus AST placebo; P<0.01) compared with estrogen-depleted AST rats receiving placebo. In contrast, cardiac mass remained elevated in rats treated with E2 plus MPA (24±5.5% versus AST+E2; P<0.01). The combination of drosperone and 17β-estradiol reduced relative heart weight to near-baseline levels. Very similar results were obtained for absolute heart weight. Systolic and mean
Global and Hemodynamic Measurements

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<th>Variable</th>
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<th>Ovx npx (n=5)</th>
<th>Ovx AST Placebo (n=10)</th>
<th>Ovx AST (n=10)</th>
<th>Ovx AST + E2 (n=10)</th>
<th>Ovx AST + E2 + spironolactone (n=10)</th>
<th>Ovx AST + E2 + drospirenone 3 mg (n=10)</th>
<th>Ovx AST + E2 + drospirenone 9 mg (n=10)</th>
<th>Ovx AST + E2 + MPA (n=5)</th>
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<td>176 ± 2*</td>
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<td>217 ± 14*</td>
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<td>533 ± 23†</td>
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<td>418 ± 11</td>
<td>361 ± 14</td>
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<td>367 ± 12</td>
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nd indicates not done; dro, drospirenone; spiro, spironolactone; BP, blood pressure.

*P<0.05 vs sham placebo; †P<0.05 vs oxv AST + E2.

Blood pressure levels were significantly elevated in ovx, AST rats compared with sham-operated or ovx/npx rats. Blood pressure levels were lower in intact AST animals and in AST rats receiving 17β-estradiol (−15±2% and −15±3% versus placebo; both P<0.001). Combining drospirenone or spironolactone with E2 substitution caused a further reduction of blood pressure, whereas MPA treatment resulted in increased blood pressure in E2-substituted rats. Serum Ang II levels were lower in ovx AST rats compared with sham-operated or ovx/npx animals (−90±2.5% versus sham; P<0.001) and not affected by E2 or MPA treatment, whereas drospirenone and spironolactone prevented the suppression of serum Ang II levels. Uterus atrophy was observed in ovx rats but not in sham-operated or E2-substituted animals. Uterus weight was lower in estrogen-substituted rats receiving drospirenone, unaltered by spironolactone, and increased in MPA-treated AST rats. Estrogen serum levels were low in ovx and increased to physiological levels in E2-substituted rats. Endometrial epithelium height was significantly lower in rats receiving E2 plus MPA or drospirenone compared with unopposed estrogen substitution. Body weight was higher in estrogen-depleted animals and decreased on E2 substitution. Heart rate was similar and within physiological limits among all of the animals.

Cardiac Myocyte Cross-Sectional Area

Cardiac hypertrophy and a patchy pattern of perivascular fibrosis were evident in AST rats from the gross morphological appearance of cardiac cross-sections (Figure 1A and 1B). The average cardiac myocyte cross-sectional area was elevated in AST compared with sham-operated rats (+128±12% versus sham; P<0.001; Figure 2) and decreased substantially with E2 substitution (−57±1.3% versus AST placebo; P<0.001) and, to a lesser extent in intact, non-ovx animals. No additional effect was observed in groups receiving E2 plus drospirenone or spironolactone. MPA blocked the decrease of the cardiac myocyte cross-sectional area in estrogen-substituted rats (+45±5% versus AST-E2) but, by itself, did not aggravate myocyte hypertrophy.

Perivascular Collagen Accumulation

AST resulted in significantly higher levels of perivascular collagen deposition compared with sham treatment or uninephrectomy ovariectomy (+300±33% versus sham; P<0.001; Figure 2).
Figure 3). E2 treatment completely blocked perivascular fibrosis in AST rats \((-277\pm 31\% \text{ versus AST-placebo}; P<0.001\) ), and no further reduction of collagen deposition was observed in animals cotreated with E2 plus drospirenone or spironolactone. No significant perivascular fibrosis was observed in intact rats receiving AST treatment. MPA aggravated perivascular fibrotic lesions and blocked the inhibitory effect of 17β-estradiol on perivascular fibrosis.

Aortic Intima:Media Ratios
Aortic intima:media ratios increased in placebo-treated AST rats compared with sham-operated or ovx and uni-npx animals (Figure 4). Combining estrogen substitution with AST treatment resulted in reduced intima:media ratios that nearly reached physiological levels. Similar observations were made in intact, non-ovx animals receiving AST treatment. No additional effect on vascular remodeling was observed in animals receiving 17β-estradiol plus drospirenone or spironolactone. The highest intima:media ratios were observed in AST rats receiving E2 plus MPA.

Vascular Osteopontin Expression
Osteopontin expression was higher in aortic specimens of ovx AST rats compared with sham-operated or ovx and uni-npx animals (Figure 5). 17β-Estradiol effectively blocked the local accumulation of osteopontin in the aortic media and intima layer. Similar results were obtained in intact animals and in ovx animals receiving E2 substitution combined with drospirenone or spironolactone. In contrast, MPA blocked the reduction of vascular osteopontin expression in estrogen-substituted AST rats but, by itself, did not increase aortic osteopontin content.

Vasomotor Studies in Isolated Aortic Rings
The contractile response of isolated aortic rings toward norepinephrine treatment was increased, and ACH-induced vascular relaxation was impaired in AST compared with sham-operated rats (Figure 6A and 6B). MPA strikingly aggravated NO-dependent vascular dysfunction as judged by impaired relaxation
to ACH treatment of aortic rings (Figure 6B). Combined treatment with 17β-estradiol plus drospirenone markedly improved endothelium-dependent relaxation to ACH compared with rats substituted with 17β-estradiol (P<0.05 versus AST; Figure 6C). In contrast, MPA treatment resulted in impaired endothelium-dependent relaxation (P<0.05 versus AST +E2 and P<0.01 versus all other groups; Figure 6C). Relaxation to ACH was blunted in all of the groups after preincubation with Nω-nitro-L-arginine methyl ester (data not shown). In contrast to endothelium-dependent relaxation, maximum endothelin-independent relaxations to the NO donor SNP were comparable in all of the groups apart from MPA-treated animals in which even endotheliun-independent relaxation was significantly impaired (Figure 6D).

Discussion

The major findings of this study can be summarized as follows: (1) 17β-estradiol substitution similar to sham ovariectomy attenuates aldosterone salt-induced hypertension, cardiac hypertrophy, vascular fibrosis, and osteopontin expression, as well as endothelial dysfunction in estrogen-depleted rats; (2) the protective effects of 17β-estradiol are abrogated by MPA; (3) MPA aggravates aldosterone salt-mediated vascular fibrosis and dysfunction; and (4) drospirenone exhibits favorable effects on cardiovascular morphology, function, and vascular osteopontin expression.

The effects of AST on cardiovascular pathology have so far only been studied in male but not in female rats. Therefore, it is interesting to note that signs of disproportionate MR activation, such as cardiac hypertrophy, hypertension, perivascular collagen accumulation, and endothelial dysfunction, as well as increased vascular osteopontin expression, were observed predominantly in ovx AST rats, whereas their severity was significantly reduced in AST rats receiving either sham ovariectomy or ovariectomy plus 17β-estradiol substitution. One important finding reported here is, therefore, the observation that endogenous estrogens, as well as substitution with physiological doses of 17β-estradiol, completely blocked or significantly improved several key features of cardiovascular injury under the condition of disproportionate MR activity.

Although the complex phenotype of AST rats suggests multifactorial mechanisms through which estrogens might attenuate cardiovascular injury and dysfunction, 17β-estradiol could be discussed to interfere directly with MR activation. However, this hypothesis is neither supported by the current literature nor by measurements of Ang II serum levels, which were suppressed in AST rats irrespective of E2 substitution, whereas MR antagonist treatment with spironolactone and drospirenone resulted in increased serum Ang II levels. Therefore, it appears more likely that 17β-estradiol interacts with downstream signal transduction pathways that become activated on AST in different organ systems. Estrogen effects are mediated by 2 different receptors,
ERα and ERβ, which confer redundant, as well as specific, effects in the cardiovascular system. The current study used the nonselective estrogen receptor agonist 17β-estradiol. Further studies that are currently underway in our laboratory will, thus, be required to determine whether selective ERα and/or ERβ agonists also attenuate cardiovascular injury in AST rats.

The extent of vascular injury in AST rats was closely linked to vascular osteopontin expression levels. Osteopontin plays an important role in cardiac and vascular fibrosis, because targeted deletion of osteopontin ameliorates cardiovascular collagen accumulation after myocardial infarction, aortic banding, and chronic Ang II infusion. Decreased osteopontin expression in estrogen-substituted AST rats thus appears as a likely mechanism to explain the lower perivascular collagen accumulation and fibrosis. Therefore, it is interesting to note that estrogens increase osteopontin expression under in vitro conditions and that ERα directly binds and transactivates the osteopontin promoter in rat osteosarcoma cells. The differential expression pattern of osteopontin under in vitro and in vivo conditions is most likely explained by very different experimental conditions. Specific studies will be required to characterize the mechanisms by which mineralocorticoids and estrogens regulate vascular osteopontin expression in vivo.

In parallel to vascular osteopontin expression, impaired ACH-induced relaxation of isolated aortic rings from ovx AST rats improved on estrogen substitution. These observations indicate that decreased NO bioavailability in ovx rats improves with estrogen substitution. Similar observations have been made previously in estrogen-depleted SHR rats and have been linked to ERα expression in ERKO mice.

The second and functionally important finding of this study is the observation that MPA almost completely blocked the protective function of 17β-estradiol in AST rats. Unfavorable effects of MPA in AST rats parallel previous reports in which progestins abrogated the reduction of vascular injury after...
The observation that MPA attenuated the protective effect of 17β-estradiol in AST rats could be explained either by enhanced mineralocorticoid activity or, alternatively, by inhibition of estrogen action. To discriminate between these mechanisms, we analyzed the cardiovascular phenotype of ovx AST rats on long-term administration of MPA. Aggravation of perivascular fibrosis and endothelium-dependent vasorelaxation in MPA-treated AST rats strongly suggest an enhancement of vascular remodeling and dysfunction. Because long-term androgen treatment in rats results in cardiac damage and hypertension via an indirect mineralocorticoid activity, we cannot exclude that the androgenic partial activity of MPA may have contributed to impaired vascular relaxation and perivascular fibrosis.

Drospirenone, which is a progestin with potent MR antagonist properties and without additional androgenic and glucocorticoid activity, might exhibit different and protective effects on cardiovascular injury in E2-substituted AST rats as compared with MPA. If this hypothesis was true, cardiac and vascular damage, vascular reactivity, and osteopontin expression should either be unaffected or even improved in drospirenone-treated AST rats. The current results strongly support this hypothesis, because drospirenone at all of the tested dosages conferred more favorable and protective effects on cardiac hypertrophy, vascular inflammation, endothelial dysfunction, and osteopontin expression under conditions of disproportionate MR activation than MPA. The observation, that drospirenone caused a further reduction of blood pressure and cardiac mass in estrogen-substituted rats is in line with recent findings in postmenopausal women. Clinical studies have confirmed the protective function of drospirenone in hypertension and cardiac hypertrophy, although the magnitude of these effects was more moderate in human studies. These differences are most likely explained by the fact that we used a model of cardiovascular injury that is strictly aldosterone dependent, whereas aldosterone levels might not have been elevated among a significant proportion of patients who were enrolled in clinical studies on drospirenone. In addition, it should be noted that, in contrast to the human situation, 17β-estradiol already caused a significant reduction of blood pressure and cardiac mass in AST rats.

**Perspectives**

We propose that novel strategies of hormone replacement therapy, including progesterone with antimineralocorticoid activity, such as drospirenone, could be advantageous compared with MPA.

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**Disclosures**

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