A comparison of ovariectomy models for estrogen studies

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Davidge, Sandra T., Yunlong Zhang, and Ken G. Stewart. A comparison of ovariectomy models for estrogen studies. Am J Physiol Regulatory Integrative Comp Physiol 280: R904–R907, 2001.—Many estrogen-replacement studies use ovariectomized animals as controls. However, ovariectomy greatly increases body weight and can enhance the peripheral synthesis of estrogen. Tamoxifen is commonly used as an antiestrogen, but it may elicit mixed agonist or antagonist actions. The aim of our study was to compare vascular function in mesenteric arteries among groups of rats with low estradiol levels. The groups (n = 5, each) of Sprague-Dawley rats were cycling (diestrus), ovariectomized (OVX), OVX + tamoxifen (OVX-T), OVX + 4-hydroxyandrostene-3,17-dione, an aromatase inhibitor (OVX-A) to prevent peripheral synthesis of estrogen, and control-fed OVX to prevent excess weight gain. Body weight was significantly elevated in only the non-control-fed OVX group. Estrogen levels were significantly greater in the cycling rats compared with the other groups, whereas uterine weights were significantly reduced in only the OVX-A and control-fed OVX groups. Methacholine relaxation was blunted only in the OVX-A and control-fed OVX groups, suggesting a possible estrogenic influence in the non-control-fed OVX and OVX-T groups. These data indicate the potential for confounding factors to decrease the efficacy of OVX controls.

Ovariectomy is a ubiquitously used model to reduce the production and influence of estrogen. However, it induces hyperphagia that, in turn, results in substantial weight gain (5). This increase in body mass constitutes a considerable source of estrogen, because peripheral tissue is capable of converting adrenal-synthesized androgen precursors into estrogen (3, 13). Consequently, it is difficult to make conclusions regarding the effects of estrogen when the apparent control conditions, which are intended to represent a state of estrogen withdrawal, actually contain substantial levels of estrogen. Indeed, ovariectomized animals are still capable of estrogen synthesis in peripheral tissues because aromatase, the terminal enzyme in the estrogen biosynthetic pathway, is expressed in many tissues (3, 6).

Treatment with tamoxifen is another method of reducing the influence of estrogen. However, tamoxifen is also known to function as a partial agonist in certain physiological conditions and cell types (11, 12). The effect of SERMs is determined by a complex interaction between factors including estrogen receptor (ER) subtypes, adaptor protein expression, ligand identity, and transcription target sites, which vary with cell type (11). Thus, similar to ovariectomy, tamoxifen may not completely abolish the influence of estrogen in vivo.

Therefore, the purpose of this study was to evaluate the validity of various models of estrogen inhibition that are used as control conditions in estrogen studies. The extensive investigation and established impact of estrogen on the vasculature suggest that evaluating the effectiveness of methods of estrogen antagonism is important to the study of vascular physiology. With the use of rat mesenteric arteries, we analyzed four groups of ovariectomized rats: ovariectomized and ovariectomized with concomitant treatment of either tamoxifen, aromatase inhibitor 4-hydroxyandrostene-3,17-dione (4-OHA), or a calorie-controlled diet. These groups were then compared with a control group of intact
cycling rats in the diestrus phase. Measurements of these groups included body and uterine weights, plasma estrogen levels, vasorelaxation to the endothelium-dependent muscarinic agonist methacholine, and vasorelaxation to the endothelium-independent exogenous nitric oxide (NO) donor sodium nitroprusside (SNP).

METHODS

**General animal model.** Female Sprague-Dawley rats (2 mo of age) were obtained from Charles River and housed in the facilities at the University of Alberta. Four groups of rats were ovariectomized (n = 5, each group) and treated with one of the following: a subcutaneous implant of a placebo pellet, a tamoxifen pellet (5 mg/pellet, 60-day release), an aromatase inhibitor, 4-OHA (350 mg/pellet, 28-day release) pellet, or a calorie-controlled (15 g of food/day) diet. Doses for the antagonist effects of the drugs were chosen based on previous reports (2, 4). Calorie-controlled ovariectomized rats were fed a quantity of food that was similar to the quantity of food consumed by the cycling animals, thereby preventing overeating. Four weeks after ovariectomy and treatment, these groups were compared with age-matched cycling rats in the diestrus phase. On the day of experiment, they were killed under light anesthesia with methohexital sodium (50 mg/kg body wt). Blood samples were collected from the right atrium, allowed to clot, and centrifuged at 3,000 rpm for 10 min. 17β-estradiol was measured by use of a radioimmunoassay kit (Diagnostic Products) The animal protocols were examined by the University of Alberta Animal Welfare Committee and found to be in compliance with the guidelines issued by the Canada Council on Animal Care.

**Experimental design.** Mesenteric arteries averaging 250 μm in diameter were chosen as a model for systemic resistance regulation as the splanchnic bed receives 30% of the cardiac output, making this vasculature an important contributor to cardiovascular homeostasis. In addition, pregnancy (a state of elevated estrogen that causes reduced vascular tone) increases mesenteric blood flow by 75% (7). Arteries were mounted in an isometric myograph system containing HEPES-buffered physiological saline solution (in mM: 142 sodium chloride, 4.7 potassium chloride, 1.17 magnesium sulfate, 1.56 calcium chloride, 1.18 potassium phosphate, 10 HEPES, and 5.5 glucose, pH 7.4). Four separate baths were used to study arterial segments simultaneously. Cumulative doses of phenylephrine (1–50 μmol/l) were administered to measure sensitivity and determine the dose that would give a 50% constriction (EC50) for each individual artery. The EC50 of phenylephrine was used to constrict arteries to achieve a baseline from which subsequent relaxation responses were measured. A dose-response curve measuring methacholine-induced relaxation was also conducted (1–1 μmol/l). After each dose-response curve had been completed, a 30-min recovery period was allowed during which the baths were changed every 10 min with fresh HEPES-buffered physiological saline solution.

Vascular smooth muscle sensitivity to the exogenous NO donor SNP (1 nM-1 μM) was measured as well. The reproducibility of repeating curves for these experiments was determined in a preliminary set of experiments designed to test for tachyphylaxis.

**Data analysis.** The data from the dose-response curves were fitted to the Hill equation, from which a straight line was generated by linear least-squares regression analysis. The EC50 was determined from this line and expressed as the geometric means ± SE. Comparison between groups was done by an analysis of variance. Post hoc analysis for comparison between groups was performed using a Tukey’s test. Differences among means were considered significant at P < 0.05.

RESULTS

**Animal model.** Animals in the ovariectomized group had significantly greater (P < 0.05) body weights than those in the three ovariectomy-plus treatment groups or the cycling group. Plasma 17β-estradiol levels were significantly higher (P < 0.05) in the cycling rats than the four ovariectomized groups that were all similar. However, uterine weight, which provides a biological marker of estrogen, was significantly lower in the 4-OHA and caloric-controlled ovariectomized rats (Table 1).

**Vascular responses.** Vasodilatory dose-response curves to methacholine were conducted on mesenteric arteries preconstricted with phenylephrine. Arteries from the cycling, ovariectomized, and tamoxifen-treated ovariectomized groups relaxed similar to the endothelium-dependent muscarinic agonist methacholine (Fig. 1). These groups demonstrated greater sensitivity to methacholine by relaxing more readily than arteries from the 4-OHA-treated ovariectomized and caloric-controlled ovariectomized groups (Fig. 1). Thus, compared with intact cycling rats, only the 4-OHA-treated ovariectomized and caloric-controlled ovariectomized groups demonstrated blunted relaxation in response to estrogen removal.

Finally, differences in vessel responsiveness to methacholine do not appear to be due to smooth muscle sensitivity, because all of the groups relaxed similarly to the exogenous NO donor SNP (Fig. 2).

DISCUSSION

In the present study, we measured vascular function to evaluate the efficacy of various methods of estrogen suppression. On the basis of vessel function as well as

### Table 1. Characteristics of animal model

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cycling</th>
<th>OVX</th>
<th>OVX-T</th>
<th>OVX-A</th>
<th>OVX-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>255 ± 4.1</td>
<td>316 ± 9.5</td>
<td>260 ± 6.1</td>
<td>224 ± 6.1</td>
<td>251 ± 4.0</td>
</tr>
<tr>
<td>E2, pg/ml</td>
<td>7.9 ± 0.3</td>
<td>5.0 ± 0.2</td>
<td>4.7 ± 0.4</td>
<td>6.0 ± 0.7</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>Uterine wt, g</td>
<td>0.33 ± 0.02</td>
<td>0.26 ± 0.04</td>
<td>0.21 ± 0.01</td>
<td>0.13 ± 0.02</td>
<td>0.11 ± 0.008</td>
</tr>
</tbody>
</table>

Values are means ± SE. Different symbols indicate P < 0.05. Groups are cycling (diestrus rats), ovariectomized (OVX), OVX + tamoxifen (OVX-T), OVX + aromatase (OVX-A), and OVX + control-fed (OVX-F). E2, 17β-estradiol.
uterine and body weights, we conclude that ovariectomized rats treated with either an aromatase inhibitor or calorie-controlled diet constitute an effective control condition for estrogen studies. However, ovariectomy models without a calorie-controlled diet or with tamoxifen treatment appear to preclude an accurate determination of the effects of estrogen.

The responses to methacholine-induced relaxation clearly demonstrate the characteristics of different estrogen-withdrawal models. The 4-OHA-treated and calorie-controlled ovariectomized rats exhibited a blunted relaxation response to methacholine. This suggests that a substantial decrease in estrogen concentration alters endothelium-dependent relaxation. In contrast, arteries from the tamoxifen-treated and non-calorie-controlled ovariectomized groups relaxed similar to the control group of cycling rats. A likely explanation for the discrepancy in vessel function among ovariectomized groups is that some of the treatments did not sufficiently suppress the estrogen pathway. Indeed, compared with the cycling (diestrus) rats, the tamoxifen-treated and non-calorie-controlled ovariec
tomized groups had similar uterine weights, whereas the uterine weights of the 4-OHA-treated and the caloric-controlled ovariectomized animals were significantly smaller, suggesting a greater inhibition of biologically active estrogens in the latter two groups.

Animals that were ovariectomized but not fed a controlled diet were significantly larger than those in the other groups. Considering that peripheral tissues, such as adipose (8) and skeletal (1, 10) muscle, are capable of synthesizing estrogen, the animals in this group likely experienced elevated estrogen levels as a consequence of the greater tissue mass. Although plasma levels of 17β-estradiol in this group were not greater than other ovariectomized groups, alternative factors could account for the proposed increase in estrogen exposure. Because our assay is specific to 17β-estradiol, several other undetected forms of estrogen could have been elevated by peripheral hormone synthesis. However, it may be that production of estrogen from peripheral tissues (i.e., from sources other than ovaries) may alter vascular function through paracrine mechanisms that may not be reflected in the plasma.

The partial agonistic effects of tamoxifen on the estrogen pathway are most likely responsible for maintaining methacholine-induced relaxation similar to that of intact cycling animals. Although tamoxifen inhibits many estrogen-mediated processes, in certain conditions such as states of low estrogen concentration (9), it is capable of binding to ERs and initiating agonistic effects. Hence, tamoxifen treatment in ovariec
tomized animals is not an effective method of attenuating the influence of estrogen. It is important to note that there are many SERMs available that we did not test in our studies, because it was not our intention to specifically evaluate efficacy of various ER blockers. The effect of SERMs is determined by a complex interaction between factors including ER subtypes, adaptor protein expression, ligand identity, and transcription target sites (11). Indeed, ICI-182,780, a commonly used estrogen antagonist, acts as an agonist through ERβ via the human retinoic acid receptor-alpha-1 promotor, whereas tamoxifen is a more potent antagonist via ERβ than ERα (14). These data further exemplify the importance of appropriate control groups for the studies of estrogen effects.

In summary, we delineated the efficacy of various models of estrogen suppression by comparing vessel function among ovariectomized groups. Relaxation curves to methacholine effectively demonstrated the differences between treatment conditions and indicated that estrogenic influences may still exist in some models of ovariectomy. Inhibiting aromatase activity with 4-OHA and controlling caloric consumption in ovariectomized animals are the two most effective paradigms for estrogen inhibition that we tested. Due to the potentially prohibitive cost of 4-OHA, controlling...
the diet of ovariectomized rats may be one of the most efficient control conditions for estrogen studies.

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REFERENCES