Inhibition of Intimal Hyperplasia Using the Selective Estrogen Receptor Modulator Raloxifene

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Hypothesis: The selective estrogen receptor modulator raloxifene hydrochloride inhibits intimal hyperplasia.

Design: Controlled laboratory experiment.

Setting: Experimental animal model.

Intervention and Main Outcome Measures: Forty-three senile female sheep were randomized to sham operation, ovariectomy, or ovariectomy followed by treatment with 17β-estradiol or raloxifene. Six months after initial operation, we performed necropsy with histological assessment of the aortic bifurcation and bone mineral densitometry and assayed serum lipids.

Results: After 6 months, serum triglyceride and total and high-density lipoprotein cholesterol levels were similar among groups and within the reference range. Ovarian ablation alone resulted in intimal hyperplasia, which was attenuated with estradiol and raloxifene therapy. Furthermore, estradiol and raloxifene therapy reversed ovariectomy-induced decreases in bone mineral density measured using spine morphometry.

Conclusions: Raloxifene attenuates ovariectomy-induced aortic intimal hyperplasia independent of serum lipid levels. These data suggest that raloxifene, in addition to its beneficial influence on bone density, has direct, beneficial cardiovascular effects.

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THE CARDIOVASCULAR protective influence of estrogen has been well documented experimentally and epidemiologically.1,2 However, nearly 50% of postmenopausal women discontinue hormone replacement therapy owing to intolerable adverse effects and safety concerns. In particular, estrogen replacement has been associated with an increased incidence of breast and endometrial cancer. Although the addition of progestins may decrease the risk for uterine neoplasia, it may reverse the cardiovascular benefits of estrogens. Therefore, the search for the optimal method of hormonal replacement continues.

Raloxifene hydrochloride is a selective estrogen receptor modulator that appears to have estrogenic effects in reversing loss of postmenopausal bone density without the risks for endometrial hyperplasia.3 Although raloxifene decreases serum cholesterol levels, the influence of raloxifene on vascular tissue in vivo remains unclear. We used the ovariecotomized ewe as a model for postmenopausal cardiovascular disease4 and demonstrated that raloxifene inhibits ovariectomy-induced aortic intimal hyperplasia independent of changes in serum lipid levels.

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Results

Basal levels of ovine estrogen are 2 to 3 pg/mL with a luteinizing peak of 8 to 10 pg/mL. The estradiol implants maintain serum levels at 4 to 6 pg/mL.7 Available estradiol assays may not accurately detect levels of less than 20 pg/mL. Therefore, LH levels were used to evaluate for completeness of ovarian ablation (Table).3 Ovariectomy resulted in an increase in LH levels from 0.6±0.4 ng/mL (0.016 ± 0.010 mIU/mL) before treatment to 2.8±0.7 ng/mL (0.072 ± 0.018 mIU/mL) after 6 months (P<.01). Treatment with estrogen or raloxifene suppressed LH release (P<.01).

To validate that our observations were independent of changes in serum lipid levels, we measured serum levels of total and high-density lipoprotein cholesterol and triglycerides (Table). Ovariectomy with or without hormone replacement did not change the lipid profile from baseline values. Levels were within accepted reference ranges and comparable to those of the sham-operation group.

As demonstrated in Figure 1, ovariectomy induced a qualitative increase in the intima as observed in the distal aorta. Hormone replacement therapy with es-
MATERIALS AND METHODS

ANIMAL MODEL

Forty-three skeletally mature, 8-year-old Warhill ewes were raised in flocks on the range and maintained on a diet of natural grass and hay. On arrival at the College of Veterinary Medicine, Colorado State University, Fort Collins, the sheep were vaccinated, wormed, shorn, and housed for a 2-week conditioning. The study design was approved by the Animal Care and Use Committee of the university. Initial procedures were performed with the sheep under general endotracheal anesthesia maintained with isoflurane and 100% oxygen. Ovariectomies and sham operations were performed using a standard ventral midline approach. A Silastic capsule was inserted subcutaneously near the iliac crest and filled with pure crystalline estradiol-17β in the animals undergoing hormone replacement treatment (OVxE group [n=7]) and with isotonic sodium chloride solution in the sham-operation animals (n=6), ovariectomy-alone animals (OVx group [n=9]), and animals undergoing ovariecxy with raloxifene treatment. Daily raloxifene hydrochloride was provided subcutaneously to 3 groups immediately after ovariectomy (OVxR group, 0.1 mg/kg per day [n=6]) and at 6 weeks after ovariectomy at a low dose (Ral-1 group, 0.02 mg/kg per day [n=8]) and at a high dose (Ral-2 group, 0.1 mg/kg per day [n=7]). Pharmacokinetically, plasma levels of raloxifene in Ral-1 and at a high dose (Ral-2 group, 0.1 mg/kg per day [n=7]). Plasma levels of raloxifene in Ral-1 and at a high dose (Ral-2 group, 0.1 mg/kg per day [n=7]).

NECROPSY AND HISTOLOGICAL ANALYSIS

Six months after initial procedures, the ewes were anesthetized and given 5000 U of heparin sodium intravenously. The animals were killed according to the guidelines set forth by the American Veterinary Medical Association Panel on Euthanasia, using 10 mL of pentobarbital sodium (400 mg/mL). We removed the aortoiliac segment and flushed it with phosphate-buffered saline. The segments were cut in 1-cm sections and immediately frozen with liquid nitrogen and stored at −70°C. We sectioned the adjacent bifurcation and fixed the sections with 10% neutral buffered formalin for 24 hours at 4°C. After paraffin embedding, 4-μm slices were placed on slides for Masson trichrome staining. To standardize the morphometric analysis, we defined the lumen, intima, and media microscopically and calculated their dimensions using a computer-linked digitizer (NIH Image 1.59b4; National Institutes of Health, Bethesda, Md). Intimal hyperplasia was expressed quantitatively by comparing the ratio of the intima to the sum of the intima and media at 6 different points as previously described.3 Triplicate measurements were performed separately by 2 blinded investigators (C.H.S. and S.A.M.) to avoid observational bias.

BONE MINERAL DENSITOMETRY

Before initial operation and necropsy, bone mineral density (BMD) of the lumbar spine was measured using a densitometer (Hologic QDR 1000/W; Hologic, Inc, Waltham, Mass) as previously described.6 Densitometry scans of the lumbar spine were measured in dorsoventral and anteroposterior views and analyzed using Hologic software (version V6.10.01; Hologic, Inc). Results are reported as percentage of change in BMD during the experimental period.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. We used analysis of variance with Bonferroni-Dunn post hoc analysis to analyze differences between individual means. Statistical significance was accepted within 93% confidence limits.

<table>
<thead>
<tr>
<th>Serum Luteinizing Hormone and Lipid Levels</th>
<th>Serum Levels</th>
<th>Animal Groups</th>
<th>Serum Luteinizing Hormone, ng/mL†</th>
<th>Total Cholesterol, mg/dL‡</th>
<th>HDL Cholesterol, mg/dL§</th>
<th>Triglycerides, mg/dL¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.5 ± 0.3</td>
<td>58 ± 5</td>
<td>38 ± 5</td>
<td>9 ± 2</td>
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</tr>
<tr>
<td>OVx</td>
<td>2.8 ± 0.7</td>
<td>56 ± 2</td>
<td>33 ± 2</td>
<td>5 ± 1</td>
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<td></td>
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<tr>
<td>OVxE</td>
<td>0.8 ± 0.4</td>
<td>67 ± 8</td>
<td>38 ± 3</td>
<td>7 ± 2</td>
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<td></td>
</tr>
<tr>
<td>Ral-1</td>
<td>0.3 ± 0.1</td>
<td>62 ± 6</td>
<td>36 ± 3</td>
<td>8 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ral-2</td>
<td>0.2 ± 0.1</td>
<td>68 ± 5</td>
<td>39 ± 4</td>
<td>16 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVxR</td>
<td>NA</td>
<td>63 ± 6</td>
<td>35 ± 4</td>
<td>16 ± 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Groups are described in the “Animal Model” subsection of the “Materials and Methods” section. Data are given as mean ± SEM. Estrogen and raloxifene hydrochloride conferred no detectable difference in lipid levels compared with sham and ovariectomy alone (OVx) animals. HDL indicates high-density lipoprotein; NA, not applicable.
†To convert to milli–international units per milliliter, multiply by 0.02586.
‡To convert to millimoles per liter, multiply by 0.02586.
§To convert to millimoles per liter, multiply by 0.01129.
¶To convert to millimoles per liter, multiply by 0.01129.
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parable with that of the sham-operation animals. Only the Ral-2 group had greatly enhanced BMD compared with the sham-operation animals (P<.01). The idea of a "magic bullet" for hormone replacement therapy, although conceptually attractive, has yet to be realized clinically. Although data exist demonstrating the beneficial effects of raloxifene on BMD without associated hyperplastic effects on uterine tissue, its influence on cardiovascular disease remains speculative. Raloxifene appears to modify serum lipid levels beneficially and to decrease serum homocysteine levels, thus indirectly influencing cardiovascular risk. Currently, long-term epidemiological studies on cardiovascular morbidity and mortality with raloxifene users are not available. Experimentally, raloxifene can inhibit vascular smooth cell proliferation, stimulate endothelial cell replication, and inhibit low-density lipoprotein oxidation in macrophages. Although these in vitro studies suggest that raloxifene can modify the response to vascular injury, the in vivo data are unclear. Two studies examined whole vessels and reached conflicting conclusions, albeit in different animals. In one, raloxifene appeared to inhibit aortic cholesterol accumulation in rabbits, whereas in the other, raloxifene had no effect on coronary artery atherosclerosis in primates.

In the present study, we demonstrated that raloxifene inhibits aortic intimal hyperplasia in the ovariectomized ewe and increases BMD independent of changes in serum lipid levels. The experimental protocol tested 2 clinical scenarios, i.e., surgical menopause with immediate raloxifene treatment and delayed treatment with low and high doses of raloxifene administered 6 weeks after ovariectomy. Estrogen and raloxifene hydrochloride replacement reversed ovariectomy-induced bone loss, and the maximal effect was seen with high-dose raloxifene (Ral-2 vs OVx groups, P<.01 [asterisk]). BMD indicates bone mineral density. Error bars represent SEM.

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animals. These data contradict a previous report that cholesterol-fed postmenopausal monkeys with higher concentrations of raloxifene increased the size of coronary plaques.\textsuperscript{13} We cannot offer a satisfying explanation for these differences outside of the particulars of the respective models. As such, our observations should be interpreted with several caveats. Although the ovariectomized ewe is an interesting model of postmenopausal cardiovascular disease,\textsuperscript{14} the histopathologic changes identified are not those of the advanced atheroma, thus limiting our conclusions correlating observations in sheep to those in humans. Aortic intimal hyperplasia at 6 months is a snapshot in time in a single vascular territory. This region was used to maximize our ability to detect vessel changes. We have not consistently identified ovariectomy-induced histological changes in gross coronary vessels. Although no coronary intimal changes were apparent, we have observed raloxifene-induced functional coronary vasoprotection as assessed by means of quantitative angiography.\textsuperscript{15} Raloxifene may provide atheroprotection without vessel remodeling. Indeed, raloxifene was recently described to promote vasomotor relaxation in rabbit coronary arteries.\textsuperscript{16} Finally, although the beneficial influence of raloxifene in the ewe is independent of alterations in lipid levels, the mechanism of vascular protection remains unknown. We have previously implicated production of peptide growth factors as important modulators of the intimal response in this model.\textsuperscript{6,17} Similar to estrogens, raloxifene likely influences the vascular response to injury at several levels.

### CONCLUSIONS

Although years of observational and experimental reports demonstrate an atheroprotective effect of estrogens, the usefulness of routine hormone replacement therapy has recently been questioned.\textsuperscript{18} The Heart and Estrogen/progestin Replacement Study was a secondary prevention study evaluating conjugated estrogen (Premarin) and progesterone replacement in postmenopausal women with known coronary disease and an intact uterus. Although no overall cardiovascular benefit was noted, this trial was limited by short follow-up (4.1 years) and the presence of coronary disease. The Women's Health Initiative is planning to randomize 27,000 women without coronary disease and to assess cardiovascular events during a 9-year period (results to be reported in 2005). Although this primary prevention trial should address many issues, physicians and their patients must struggle with balancing the risks and benefits of estrogen replacement therapy. Much enthusiasm now exists for the creation of an “ideal” estrogen. Raloxifene is only one of several selective estrogen receptor modulators available. Clinically, raloxifene has been approved by the US Food and Drug Administration for the treatment of osteoporosis and appears to protect postmenopausal women from breast cancer.\textsuperscript{19} Will raloxifene provide atheroprotection? The Raloxifene Use for the Heart study plans to evaluate raloxifene as a primary and secondary coronary prevention agent in 10,000 postmenopausal women.\textsuperscript{20} Experimentally, our data suggest that raloxifene may directly influence the vessel wall in vivo. Cumulatively, selective estrogen receptor modulation may offer targeted hormone replacement therapy, by avoiding some of the deleterious effects of estradiol while providing similar cardiovascular protection.

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### REFERENCES