17β-Estradiol Reduces Glycoxidative Damage in the Artery Wall

Barbara A. Walsh, Bonnie L. Busch, Adam E. Mullick, Karen M. Reiser, John C. Rutledge

Abstract—Glycoxidative damage in the vasculature has been linked to atherosclerotic cardiovascular disease. Estrogens protect against the development and progression of atherosclerosis. Because estrogens are potent antioxidants that also effect glucose metabolism, part of their protection against atherosclerosis could be through attenuation of glycoxidative damage in the vascular wall. In this study, we tested the hypothesis that chronic estradiol administration is associated with decreased levels of glycoxidative damage in arterial walls. We harvested and examined iliac arteries from ovariectomized, 8-month-old rats that had been implanted for 6 months with 1 of the following subcutaneous hormone pellets: low estradiol (2.5 mg estradiol), high estradiol (25 mg estradiol), P₄ (200 mg progesterone), low estradiol and P₄, placebo (no hormone), or control (no implant). Using pentosidine as a biomarker of glycoxidative damage, we found that all vessels from rats receiving estradiol (low estradiol, high estradiol, and low estradiol + P₄) exhibited a 50% reduction in glycoxidative damage compared with P₄, placebo, and control vessels (P<0.05). Consistent with this finding, we observed that estradiol-treated rats had a 30% decrease in tissue levels of hydroperoxides, a marker of oxidative stress. Finally, estradiol-treated rats had a small, but significant, decrease in plasma glucose levels (P<0.01).

In summary, we report the novel finding that chronic estrogen administration is associated with significant decreases in glycoxidative damage and oxidative stress in the arterial wall. It seems likely that these actions may constitute a mechanism by which estrogen attenuates the progression of atherosclerosis.

Key Words: estrogen ■ glycoxidative damage ■ oxidant stress ■ glycemic stress ■ atherosclerosis

Glycoxidative damage is a general term often used to describe the progressive accumulation of reaction products resulting from nonenzymatic glycation and oxidation in tissues. Several of these reaction products, such as pentosidine and carboxymethyllysine, have now been structurally identified. Accumulation of these glycoxidative products on matrix proteins is observed in normal physiological processes such as aging as well as in a number of pathophysiological processes, including atherosclerosis and diabetes mellitus.

Accumulation of glycoxidation products in the arterial matrix may disrupt normal vascular function. Altered physicochemical properties of arterial matrix molecules are described in atherosclerosis. Glycoxidation products have been reported to affect permeability and/or macromolecule accumulation in endothelial cell cultures and blood vessels. Furthermore, in vitro studies suggest that glycoxidative damage may be responsible for promoting increased LDL binding in the artery wall. Recent data in primates found that chronic 17β-estradiol supplementation decreased LDL accumulation in vivo. It is possible that estradiol protects against LDL accumulation by attenuating the production of glycoxidative products by diminishing oxidant and/or glycemic stress.

Recent research supports an antioxidant role for estradiol. Both biochemical and cell culture studies indicate that estradiol acts as a scavenger of oxygen radicals as well as a chain-breaking antioxidant. Additionally, estradiol has been shown to provide antioxidant protection to lipoprotein particles as well as membranes and tissues. Therefore, 1 mechanism by which estradiol could decrease glycoxidative damage in the artery wall is through antioxidation protection of the vascular tissue.

Alternatively, estradiol could protect against the accumulation of glycoxidative products by diminishing glycemic stress. Andersson et al found that postmenopausal women receiving oral estrogens for 3 months exhibited decreased blood glucose and glycosylated hemoglobin levels. In addition, Brussard et al observed that short-term oral estrogen therapy improved insulin resistance in postmenopausal women with non–insulin-dependent diabetes. These studies suggest that estradiol could attenuate the process of nonenzymatic glycation through improved glucose homeostasis.

We undertook this study to examine the effects of chronic estradiol supplementation on glycoxidative damage in the artery wall. To accomplish this goal, an ovariectomized rat...
model implanted with female sex hormones was developed, and we used this model to (1) describe the effects of estradiol on markers of glycoxidative damage and oxidative stress in the arterial wall and (2) examine the potential mechanisms responsible for these differences.

**Methods**

**Animal Care and Hormone Supplementation**

Ovariectomized female Crl:CD (SD)BR rats (5 weeks old) were purchased from Charles River Laboratories (Boston, Mass) and maintained in a facility approved by the Animal Use Committee at the University of California, Davis. Animals received rat chow and water ad libitum and were kept on a 12:12-hour light/dark cycle. All protocols were approved by the Animal Use and Care Administration Advisory Committee.

Pellets containing ovarian sex hormones (Innovative Research, 90-day-release pellets) were implanted in these rats 3 to 4 weeks after their arrival. The animals were anesthetized intraperitoneally with xylazine (10 mg/kg) and ketamine (80 mg/kg). A posterior longitudinal 1.0-cm-long skin incision was made in the neck, starting from the base of the skull. Underlying muscle tissue was separated from the skin, and care was taken to not tear the muscle or surrounding fascia. Pellets contained 1 of the following: low estradiol (2.5 mg estradiol), high estradiol (25 mg estradiol), P₄ (200 mg progesterone), low estradiol and P₄, placebo (no hormone), or control (no implant) and were placed in the small, lateral, subcutaneous spaces posterior to the ears. The dorsal skin incision was sealed with Nexaband tissue glue, and the animals were given an intramuscular injection of the antibiotic enrofloxacin (10 mg/kg) and were placed on a heating pad for recovery. This procedure was repeated after 90 days (total of 180 days of treatment).

Animals were fasted for 12 hours before tissue collection. On the experimental day, animals were anesthetized intraperitoneally with 65 mg/100 mg body weight of 50 mg/mL sodium pentobarbital. Blood for plasma analysis of all hormone and glucose levels was immediately collected. Vascular tissues (aortas and iliac arteries) were extracted directly after exsanguination.

**Plasma Assays**

Blood was collected from each animal through the right atrium by using a 22-gauge needle and a heparinized syringe. Blood was transferred to sterile Vacutainers (Becton Dickinson) and centrifuged (2800 rpm for 10 minutes). Plasma samples were separated from blood cells and kept at −20°C until assayed for the following components discussed below.

**Estradiol and Progesterone Levels**

Plasma samples were sent to the University of California at Davis Endocrinology Laboratory for radioimmunoassay analysis of estradiol concentration and ELISA of progesterone concentration.

**Glucose and Insulin Levels**

Plasma glucose was measured using a Yellow Springs Instruments 2300 STAT Plus glucose analyzer. Plasma insulin was measured by the technique of Yallow and Berson, modified by use of a 0.05 mol/L phosphate buffer containing 0.4% human serum albumin (Cutter Biological) and a PEG method. Chemicals were obtained from the following sources: PEG from Sigma Chemical Co; rat insulin standard (23.1 U/mg) from Novo Biolab; insulin antisera (porcine) from ICN Diagnostics; and 125I-labeled insulin from Amersham.

**Tissue Assays**

Immediately after exsanguination, the aorta and both right and left iliac arteries were removed, rinsed in Krebs’ buffer solution, and stored at −80°C until use. The aorta was used for analysis of catalase gene expression. The 2 iliac arteries were analyzed for pentosidine and hydroperoxide content after they had been pooled together, lyophilized, hydrolyzed in 6N HCl for 24 hours, methanol-extracted, evaporated under N₂ gas, and resuspended in double-distilled water. These tissue assays were performed as described below:

**Pentosidine Quantification**

Pentosidine is a standard and specific marker of extracellular matrix glycation. This fluorophore consists of single lysine and arginine moieties cross-linked to a pentose. Pentosidine was assayed as described previously. In brief, a standard was prepared as follows. After incubation of (1 mmol/L each) arginine, ribose, and lysine, the sample mixture was filtered and washed with water and pyridine before elution of the pentosidine-enriched fraction with NaOH. After the pH was adjusted to 7.4, the material was concentrated and chromatographed. Fractions containing the fluorophore were pooled, adjusted to pH 8.5, and dried. The pentosidine was again extracted and repeatedly chromatographed by reverse-phase high-performance liquid chromatography using a water-acetonitrile gradient containing n-heptafluorobutyric acid and trifluoroacetic acid. A solution of standard concentration was prepared after purification and lyophilization of the fluorophore. The identity of the standard was further confirmed by autofluorescence detection (emission = 335/excitation = 385) at pH 2, 7, and 9. Aliquots of the standard were used at the start and end of each assay period.

Samples were analyzed by high-performance liquid chromatography (see Figure 1) at a flow rate of 1 mL/min and using a linear gradient of 0% to 17% acetonitrile containing n-heptafluorobutyric acid as the counterion. Autofluorescence detection was captured at an excitation wavelength of 330 nm and an emission wavelength of 380 nm by using a Xe bulb–equipped Hitachi model D fluorometer. A model D-2000 Hitachi integrator was used to integrate peak areas. Routine calibration experiments were performed, such as analysis of a tissue standard (human trachea), that contains high levels of pentosidine, and chromatography of the purified standard (Figure 1) at the start and end of each assay period. Pentosidine values were...
expressed relative to collagen content (in the units of picomoles of pentosidine per nanomoles of collagen) after colorimetric determination of hydroxyproline content of the sample hydrolysates.

### Tissue Hydroperoxide Quantification

Tissue hydroperoxide levels were measured by the PeroXO Quantitative Ribonuclease Protection Assay (Pierce). We chose this method because of its simplicity and ability to detect nanomolar quantities of hydroperoxides under physiological conditions.22 In brief, 40 μL of water-solubilized sample was added to 360 μL of a mixture containing the following: 2.5 mM NaCl, 0.25 mM CaCl₂, 100 mM sorbitol, and 125 μM xylene orange. The reaction was incubated at room temperature for 30 minutes and absorbance read at 560 nm in a spectrophotometer. Peroxides were expressed relative to total protein content (in the units of picomoles of hydroperoxides per microgram protein) after determination of total protein (Pierce, modified Lowry protein assay).

### Catalase Gene Expression

The effect of female sex hormones on catalase gene expression was assessed in the aortas of hormone-supplemented animals by utilizing a ribonuclease protection assay (RPA). We chose to examine the antioxidant enzyme catalase because it has been implicated in atherosclerosis.23 First, a partial cDNA catalase clone was synthesized from total liver RNA by polymerase chain reaction amplification of hydroxyproline content of the sample hydrolysates.

#### Terminal Body Weight and Plasma Concentrations of Estradiol (E₂), Glucose, and Insulin in 8-Month-Old Rats After 6 Months of Chronic Hormone Supplementation

<table>
<thead>
<tr>
<th>Hormone Treatment (n=5 or 6 Animals/Group)</th>
<th>E₂ pg/mL</th>
<th>Glucose mMol/L</th>
<th>Insulin pmol/L</th>
<th>Terminal Body Weight g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low estradiol</td>
<td>69±21†</td>
<td>6.1±0.5</td>
<td>172±37</td>
<td>356±17†</td>
</tr>
<tr>
<td>High estradiol</td>
<td>373±88*</td>
<td>5.2±0.2</td>
<td>215±50</td>
<td>323±9</td>
</tr>
<tr>
<td>Low estradiol+P₄</td>
<td>23±8†</td>
<td>6.3±0.5</td>
<td>483±148</td>
<td>375±19†</td>
</tr>
<tr>
<td>P₄</td>
<td>5±3‡</td>
<td>6.8±0.2</td>
<td>546±117</td>
<td>460±15‡</td>
</tr>
<tr>
<td>Placebo</td>
<td>6±4‡</td>
<td>6.7±0.3</td>
<td>462±145</td>
<td>461±21‡</td>
</tr>
<tr>
<td>Control</td>
<td>4±2†</td>
<td>6.9±0.6</td>
<td>393±102</td>
<td>433±21†</td>
</tr>
</tbody>
</table>

Different symbols in each column, namely, *, †, and ‡, indicate significant differences at P<0.05.

#### Results

**Plasma Hormone Levels Resulting From Various Hormone Implants**

The high-estradiol group obtained plasma levels of estradiol that were 5- to 10-fold higher than the 2 groups receiving the low dose of estradiol. All animals receiving estrogen implants obtained higher plasma levels of estradiol compared with the groups receiving placebo, P₄, or no implant (P<0.05, Table). Plasma P₄ levels were 17±4 ng/mL in the P₄ group, 15±6 ng/mL in the placebo group, 22±5 ng/mL in controls, 25±7 ng/mL in the high-estradiol group, 28±7 ng/mL in the P₄ and low-estradiol group, and 25±6 ng/mL in the low-estradiol group. All values were in the normal range, as average values in cycling rats range between 5 and 30 ng/mL.27 Statistically, there was no difference between the groups. These P₄ findings were unexpected and are explained further in the Discussion.

#### Effects of Hormones on Arterial Collagen Accumulation

We analyzed the quantity of collagen, as indicated by hydroxyproline content per unit of total protein, in all hormone treatment groups. Hydroxyproline has been used extensively to indicate the presence of collagen in tissues.20,21 Collagen (hydroxyproline) accounted for 32% to 36% of the total iliac artery protein content, with the values ranging from 0.32 to 0.36 μg hydroxyproline per microgram total protein. There were no significant differences among the groups (n=4 per group).

### Estradiol Supplementation Decreases Nonenzymatic Glycation in the Artery Wall

The effects of hormone on pentosidine were assessed in iliac arteries from 8-month-old, ovarioctomized, hormone-
implanted rats. Treatment with estradiol resulted in a 50% reduction \((P<0.05; \text{Figure 2})\) in arterial pentosidine levels compared with control and placebo treatments. The effect of estradiol on pentosidine was not affected by hormone dose or the presence of P4. Interestingly, pentosidine levels in iliac arteries from P4-treated animals exhibited intermediate levels of pentosidine \((28\% \text{ lower than control or placebo values; } P<0.05)\).

**Effects of Hormone Treatment on Vascular Wall Tissue Hydroperoxide Levels**

The effects of hormones on hydroperoxide levels were assessed in iliac arteries from 8-month-old, ovariectomized, hormone-implanted rats. Vessels from estradiol-treated rats had decreased levels of tissue peroxide \((\text{in picomoles per microgram tissue protein): high estradiol, } 0.47 \pm 0.06; \text{low estradiol and } P_4, 0.52 \pm 0.06; \text{low estradiol, } 0.54 \pm 0.06; P_4, 0.64 \pm 0.08; \text{placebo, } 0.67 \pm 0.08; \text{and control, } 0.69 \pm 0.05 \text{ } (P=0.116)\). When vessels were grouped for an overall treatment effect, it was noted that peroxide levels in all estradiol-treated vessels \((\text{high estradiol, low estradiol, and low estradiol+P4})\) were 30% lower than those seen in the non-estradiol-treated vessels \((P<0.01; \text{Figure 3})\).

**Hormone Effects on Body Weight**

Hormone supplementation altered body weight gain in ovariectomized rats \((\text{Table})\). Control, P4-implanted, and placebo-implanted rats increased their body weight by 28% to 30% from the time of the first implant until the experiment date \((5 \text{ to 6 months later})\). Rats implanted with low estradiol and low estradiol with P4 displayed an 8% to 10% increase in body weight, whereas the high-estradiol group actually decreased their body weight by 10% \((P<0.05; \text{Table})\). Initial mean body weights for the various treatment groups ranged from 320 to 360 g and were not statistically different from each other \((P=0.216)\).

**Effects of Estradiol Treatment on Plasma Glucose, Insulin, Triglyceride, and Cholesterol**

Hormone implants altered glucose and insulin levels \((\text{Table})\). Both plasma glucose and insulin tended to be lower in the

**Effects of Chronic Hormone Supplementation on Catalase Gene Expression**

We hypothesized that the control, P4-implanted, and placebo-implanted animals would exhibit elevated levels of arterial antioxidant gene expression due to the raised levels of oxidant stress \((\text{H}_2\text{O}_2)\). Consequently, catalase gene expression was measured in aortas from animals after the 5 to 6 months of hormone supplementation \((n=4 \text{ animals per group})\). The P4 treatment group exhibited a slight, but insignificant, increase in the level of catalase expression \((P=0.16; \text{Figure 4})\). Levels of catalase expression in ascending order were as follows: high estradiol, 69/74; low estradiol, 82/137; low estradiol with P4, 53/87; P4, 54/95; placebo, 52/82; and control, 52/75.

**Discussion**

Our studies are the first to describe an effect of estradiol on glycoxidative damage in the artery wall. Chronic estradiol
supplementation resulted in decreased levels of oxidative stress and glycoxidative damage in the rat vasculature. These findings are important in that they describe potential alternative mechanisms involved in the antiatherogenic protection of estradiol.

We were able to obtain physiological levels of plasma estradiol in our experimental animals by using subcutaneous hormone implants. The high-estradiol group achieved plasma estradiol levels that are seen in pregnant rats, and both of the low-estradiol groups (low estradiol and low estradiol + P<sub>4</sub>) obtained plasma estradiol levels that are seen in intact, cycling rats. Our plasma P<sub>4</sub> levels resulting from the implants were surprising. Although all were in the normal range (15 to 28 ng/mL), the values were higher than would be expected with unopposed P<sub>4</sub> to exhibit increased catalase gene expression, because aortic catalase levels were 55% to 65% higher in this group compared with all other treatment groups.

Estradiol supplementation resulted in decreased levels of oxidative stress and glycoxidative damage in the rat vasculature. These findings are important in that they describe potential alternative mechanisms involved in the antiatherogenic protection of estradiol.

Changes in aortic catalase gene expression resulting from chronic hormone supplementation. Catalase gene expression in each aorta (n=4 animals per group) was assessed by the RPA and reported relative to β-actin expression (unitless number). There was a tendency (P=0.16) for aortas from animals treated with unopposed P<sub>4</sub> to exhibit increased catalase gene expression, because aortic catalase levels were 55% to 65% higher in this group compared with all other treatment groups.

Estradiol supplementation resulted in less (low estradiol) or no (high estradiol) weight gain, compared with controls, from the time that the rats were initially implanted at 3 months of age until they were killed at 8 to 9 months of age. It is well established that estradiol alters behaviors that determine body weight in rats (for a review, see Wade<sup>34</sup>). Specifically, estradiol increases activity and decreases food intake,<sup>35,36</sup> both of which favor a reduction in fat stores. Other mechanisms involved in estradiol’s effects on body weight have been suggested, such as lowering of the “lipostat” setpoint in the hypothalamus<sup>37</sup> and increasing the sympathetic nervous system activity to white adipose tissue.<sup>38</sup> Thus, estradiol may act through a variety of mechanisms to alter body weight.

The decreased levels of plasma insulin and glucose seen with estradiol treatment are at least partially due to the altered body weight and adiposity, because our results showed that body weight was a significant predictor of plasma glucose levels. Increased plasma levels of insulin suggest increased insulin resistance, a phenomenon correlated to increased body weight, specifically, increased (android) body fat<sup>39</sup>. This phenomenon is seen after menopause in women<sup>40</sup> and after ovariectomy in rats.<sup>41</sup> We also observed this in our ovariectomized rats. Alternatively, a direct role of estrogen on insulin production and action and glucose utilization, independent of adiposity, cannot be ruled out. Estradiol has been shown to increase insulin secretion and augment B-cell function<sup>42,43</sup> as well as decrease hepatic glucose production.<sup>44</sup> Furthermore, ovariectomy in rats results in decreased glucose uptake as well as diminished glycogen synthesis in muscles,<sup>45</sup> independent of changes in body weight. It is apparent that estradiol may act through a variety of mechanisms to alter levels of plasma insulin and glucose.

The 18% decrease in glucose levels observed in the estrogen-treated rats may have played a role in the vascular accumulation of glycoxidative products over time. The course of glycoxidative damage is determined by both time and glucose concentration.<sup>4</sup> For instance, nonenzymatic protein glycation occurs under normal glycemic conditions with aging as well as during short-term exposure to hyperglycemia, as seen in diabetes mellitus. Therefore, 5 to 6 months of vascular exposure to elevated levels of plasma glucose seen in the non–estradiol-treated rats, though still in the physiological range, could explain the elevated levels of glycoxidative damage to these iliac arteries.

It is unclear whether the decreased arterial wall hydroperoxide levels in arteries from estradiol-implanted rats were due to an antioxidant effect of estradiol on the artery wall or secondary to decreased glyceric stress. Estradiol has been shown to confer direct antioxidant protection against oxygen radicals<sup>8,12</sup> and to alter levels of antioxidant enzymes.<sup>46,47</sup> Therefore, it is possible that estradiol had an antioxidant effect on the artery wall. This concept would imply that estradiol, through its antioxidant activity, diminished oxida-
tive stress (hydroperoxide) in the artery wall. Conversely, improved plasma glucose levels could result in decreased tissue peroxide levels. Wolff and Dean\(^\text{18}\) have shown that autoxidation of glucose addsucts leads to the production of reactive oxygen species (H\(_2\)O\(_2\)) and protein modification. Later, this same group\(^\text{50,51}\) described the generation of H\(_2\)O\(_2\) during protein incubation with glucose. On the basis of these observations, one could argue that the decreased levels of hydroperoxide seen in vessels from estradiol-treated rats were due to the diminished glucose levels obtained with estradiol replacement. Future studies are planned to distinguish between the primary and secondary effects of estradiol on arterial oxidative stress.

Oxidant stress is known to increase expression of antioxidant enzymes.\(^\text{50,51}\) Because arterial tissue from control, placebo-implanted, and P\(_4\)-implanted rats exhibited a 1.3-fold increase in H\(_2\)O\(_2\), we hypothesized that aortic tissue from these same groups would also display elevated levels of catalase gene expression. Although we found no significant difference between these tissues and those from the estradiol-treated rats, this finding does not rule out the possibility of a vascular tissue response to oxidant stress in these animals. Others have shown increased measures of alternative oxidative stress markers, such as gene expressions of heme oxygenase-1, as well as activation of the oxidant stress–sensitive transcription factor nuclear factor-\(\kappaB\).\(^\text{52,53}\) Clearly, this combination of oxidative and glyemic stress and sex hormones is very complex. Much work needs to be performed to elucidate mechanisms involved in the vascular response to these conditions.

The specific effects of estrogen replacement therapy on insulin sensitivity, glycemic control, body weight, and fat distribution in nondiabetic, menopausal women themselves are inconsistent. Rats are susceptible to decreased weight gain due to the diminished glucose levels obtained with estradiol and P\(_4\)-implanted rats. However, Wagner et al\(^\text{55}\) have shown that primates receiving oral conjugated equine estrogens did not differ in body weight, blood glucose, or insulin from the control, ovariectomized animals. In that study, the estrogen-treated group did exhibit a statistically significant increase in body weight to a central (android) distribution in early-postmenopausal women as well.\(^\text{54}\) This result is similar to what we observed in our estradiol-implanted rats. However, Wagner et al\(^\text{55}\) have shown that primates receiving oral conjugated equine estrogens did not differ in body weight, blood glucose, or insulin from the control, ovariectomized animals. In that study, the estrogen-treated group did exhibit a statistically significant increase in skin total fluorescence and a tendency for increased aortic fluorescence. Therefore, it would appear that much more work needs to be performed in this area to determine more precisely the mechanisms involved in estrogen therapy on glucose metabolism, body weight/fat alterations, and production of glycoxidation products in both postmenopausal women and animals. Interpretation of this work will have to consider not only the model used but also the type of estrogen as well as the route of administration.

In summary, estradiol may be acting through many potential mechanisms, such as decreased body weight, insulin, and glucose, to diminish glycoxidative damage in the arterial wall. Glycoxidative damage to the artery wall is known to alter vascular function through increasing permeability,\(^\text{32}\) vascular stiffening,\(^\text{56}\) and LDL binding,\(^\text{7}\) all parameters that play a role in the progression of atherosclerosis. Therefore, our studies support a new antiatherogenic role for estradiol at the site of the artery wall. More work is needed to distinguish between the primary versus secondary effects of estradiol on these processes.

Acknowledgments

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