**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 affect 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. (Arterioscler Thromb Vasc Biol. 1999;19:840-846.)

**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 antioxidant 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, Inc) from ICN Diagnostics; and 125 I-labeled insulin from Amersham. 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.1 Pentosidine was assayed as described previously.20 In brief, a standard was prepared as follows. After incubation of (1 mmol/L each) arginine, ribose, and lysine, the fluorescent material was separated by high-performance liquid chromatography using a water-acetonitrile gradient containing 0.4% human serum albumin (Cutter Biological) and a 0.05 mol/L phosphate buffer containing 0.4% human serum albumin (Cutter Biological) and a 0.05 mol/L phosphate buffer containing 0.4% human serum albumin (Cutter Biological). The identity of the standard was further confirmed by autofluorescence detection (emission 355/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-heptfluoroobutyric 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.

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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.
expressed relative to collagen content (in the units of picomoles of pento- 
sosidine per nanomoles of collagen) after colorimetric determi-
nation of hydroxyproline content of the sample hydrolysates.

**Tissue Hydroperoxide Quantification**

Tissue hydroperoxide levels were measured by the PeroXOQuant
quantitative peroxide assay (Pierce). We chose this method because of
its simplicity and ability to detect nanomolar quantities of
hydroperoxides under physiological conditions. In brief, 40 µL of
water-solubilized sample was added to 360 µL of a mixture
containing the following: 2.5 mmol/L iron(II)sulfate, 0.25 mol/L
H₂SO₄, 100 mmol/L sorbitol, and 125 µmol/L xylene orange.
The reaction was incubated at room temperature for 30 minutes and
absorbance read at 560 nm in a spectrophotometer. Peroxide values
were expressed relative to total protein content (in the units of
picomoles hydroperoxide per microgram protein) after determination
of total protein (Pierce, modified Lowry protein assay).

**Catatalase 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. First, a partial cDNA catalase clone was synthe-
sized from total rat liver RNA by polymerase chain reaction
cloning with primer oligonucleotides based on the published
nucleotide (nt) sequence for rat liver catalase cDNA. The primer
oligonucleotides were as follows: the 5'-primer was identical to the
nt sequence for rat liver catalase cDNA, and 3'-primers, respectively. The cDNA fragment was restricted and
ligated into the multiple cloning site of the pBluescript II SK
expression vector (Stratagene).

Next, an antisense riboprobe for catalase was synthesized with use
of the MAXIScript in vitro transcription kit (Ambion). In brief, the
template DNA (as above) was linearized with the restriction enzyme
HindIII, and the riboprobe was synthesized using T7 RNA polymer-
ase and [α-³²P]UTP (NEN Life Science Products). At the same time,
a control mouse Tri-β-actin riboprobe (β-actin; 250 nt) was synthe-
sized from a cDNA fragment supplied by the manufacturer.
The template DNA was removed and the riboprophes were purified on a
common RNA sample present in all assays.

**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. 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. 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 pento- 
sosidine were assessed in iliac arteries from 8-month-old, ovariec-
tomized, hormone-
implanted rats. Treatment with estradiol resulted in a 50% reduction ($P < 0.05$; 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 $P_4$. Interestingly, pentosidine levels in iliac arteries from P$_4$-treated animals exhibited intermediate levels of pentosidine (28% 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 (in picomoles per microgram tissue protein): high estradiol, 0.47 ± 0.06; low estradiol and $P_4$, 0.52 ± 0.06; low estradiol and $P_4$, 0.54 ± 0.06; $P_4$, 0.64 ± 0.08; placebo, 0.67 ± 0.08; and control, 0.69 ± 0.05 ($P = 0.116$). When vessels were grouped for an overall treatment effect, it was noted that peroxide levels in all estradiol-treated vessels (high estradiol, low estradiol, and low estradiol + $P_4$) were 30% lower than those seen in the non-estradiol-treated vessels ($P < 0.01$; Figure 3).

**Hormone Effects on Body Weight**

Hormone supplementation altered body weight gain in ovariectomized rats (Table). Control, $P_4$-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 to 6 months later). Rats implanted with low estradiol and low estradiol with $P_4$ displayed an 8% to 10% increase in body weight, whereas the high-estradiol group actually decreased their body weight by 10% ($P < 0.05$; 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 (Table). Both plasma glucose and insulin tended to be lower in the groups receiving estrogen only when differences across all 6 groups were analyzed ($P = 0.086$ and $P = 0.14$ for glucose and insulin, respectively; by ANOVA). When the hormone treatments were grouped to observe an overall estrogen effect, we found that insulin was decreased in estradiol only–treated vessels compared with all other groups (195 ± 31 versus 470 ± 61 pmol/L; $P = 0.006$). Although glucose levels for both groups were in the normal range of values for rats, similar findings were observed in the other groups (5.67 ± 0.3 versus 6.69 ± 0.2 mmol/L for estradiol only versus all others, respectively; $P = 0.004$). Multiple linear regression analysis indicated that body weight was a significant predictor of glucose levels ($P < 0.01$), whereas estradiol ($P = 0.10$), insulin ($P = 0.50$), and $P_4$ ($P = 0.88$) did not contribute significantly.

Because almost all of the plasma was used for analysis of glucose, insulin, estradiol, and $P_4$, the remaining plasma from animals of the same treatment group was pooled, and total cholesterol and triglycerides were determined by an automatic enzymatic analyzer. Plasma levels of total cholesterol and triglycerides for all rats ranged from 52 to 82 mg/dL and from 74 to 137 mg/dL, respectively. The total cholesterol to triglyceride ratios in each group were as follows: high estradiol, 69/74; low estradiol, 82/137; low estradiol with $P_4$, 53/87; $P_4$, 54/95; placebo, 52/82; and control, 52/75.

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

We hypothesized that the control, $P_4$-implanted, and placebo-implanted animals would exhibit elevated levels of arterial antioxidant gene expression due to the raised levels of oxidant stress ($H_2 O_2$). Consequently, catalase gene expression was measured in aortas from animals after the 5 to 6 months of hormone supplementation (n = 4 animals per group). The $P_4$ treatment group exhibited a slight, but insignificant, increase in the level of catalase expression ($P = 0.16$; Figure 4). Levels of catalase expression in ascending order were as follows: high estradiol, 8.05 ± 0.03; low estradiol plus $P_4$, 0.234 ± 0.04; control, 0.246 ± 0.01; placebo, 0.25 ± 0.04; and $P_4$, 0.38 ± 0.05.

**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,28 and both of the low-estradiol groups (low estradiol and low estradiol+P4) obtained plasma estradiol levels that are seen in intact, cycling rats.27 Our plasma P4 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 ovariectomy, and there was no difference in plasma P4 values between the P4-implanted rats and the other groups in this study. We believe that the P4 values obtained here were the result of an acute stress–induced adrenal response to the handling and anesthetizing of the rats on the experimental day. The concept that the adrenal glands are capable of producing significant amounts of P4 is well documented.29,30 Resko29 and others have described a direct relationship between increased stress and adrenocorticotropic hormone levels. 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.4 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 radicals12,13 and to alter levels of antioxidant enzymes.46,47 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\(^{48}\) have shown that autoxidation of glucose adducts leads to the production of reactive oxygen species (H\(_2\)O\(_2\)) and protein modification. Later, this same group\(^{59}\) 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.\(^{50,51}\) 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-\(\kappa\)B.\(^{52,53}\) Clearly, this combination of oxidative and glycemic 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 induced by the diminished glucose levels obtained with estradiol replacement. Future studies are planned to distinguish between the primary versus secondary effects of estradiol on the site of the artery wall. More work is needed to distinguish between the primary versus secondary effects of estradiol on these processes.

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