17β-Estradiol Reduces Glycoxidative Damage in the Artery Wall

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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), P$_4$ (200 mg progesterone), low estradiol and P$_4$, 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 + P$_4$) exhibited a 50% reduction in glycoxidative damage compared with P$_4$, 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 estradiol 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, a 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. Anderson 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 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 0.5 ml of saline, as described previously. 20

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, 18 modified by use of a 0.05 mol/L phosphate buffer containing 0.4% human serum albumin (Cutter Biological) and a PEG method. 19 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.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 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-heptfluorobuturyric 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-heptfluorobutyric 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. Pentosidnue values were

Figure 1. Chromatographic assessment of pentosidine in iliac arteries from 8-month-old ovariectomized rats subjected to different hormone treatment protocols. Typical results are shown. A, Pentosidine standard, 200 pmol; B, artery from a control rat; and C, artery from a high estradiol–treated rat. Pentosidine peaks are shown in solid black, eluting at 28 minutes. Note that the pentosidine peak in the estradiol-treated rat appears smaller than that in the untreated rat. The fluorescent material eluting in the void volume, as well as peaks eluting before pentosidine, have been shown to contain no pentosidine.
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 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 absorbance read at 560 nm in a spectrophotometer). Peroxide values were normalized to the β-actin signal in each sample (ie, catalase cpm divided by β-actin cpm=unitless number) to account for any RNA loading variability. To normalize values between assays, the ratio of catalase to β-actin for each sample was divided by the corresponding ratio for a common RNA sample present in all assays.

**Statistical Analysis**

All statistical analyses were performed with SigmaStat 2.0 by Jandel Scientific Software. Hormone treatment effects were analyzed by 1-way ANOVA, and the Student-Newman-Keuls post hoc test was used to analyze for significant effects. Tests of significance were applied at the 5% level. Where appropriate, treatments were grouped to observe an overall treatment effect (eg, estradiol treatment versus no estradiol treatment) and analyzed by t test.

**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. The 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 pentosidine were assessed in iliac arteries from 8-month-old, ovariectomized, hormone-
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 (H₂O₂). 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 P₄ 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, 0.231±0.05; low estradiol plus P₄, 0.234±0.04; control, 0.235±0.04; low estradiol, 0.246±0.01; placebo, 0.25±0.04; and P₄, 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 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 P₄. Interestingly, pentosidine levels in iliac arteries from P₄-treated animals exhibited intermediate levels of pentosidine, which were not different from the estradiol or control groups. Because almost all of the plasma was used for analysis of glucose, insulin, estradiol, and P₄, 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 P₄, 53/87; P₄, 54/95; placebo, 52/82; and control, 52/75.

Effects of Chronic Hormone Supplementation on Catalase Gene Expression

Figure 2. Hormone effects on pentosidine levels in iliac arteries (n=4 animals per group). Iliac arteries from P₄-implanted rats exhibited pentosidine levels that were 50% lower than in arteries from control and placebo-implanted rats (P<0.05; different letters signify significant differences). P₄-implanted rats exhibited intermediate levels of pentosidine, which were not different from the estradiol or control groups.

Figure 3. Hydroperoxide levels measured in vessels from rats receiving high estradiol, low estradiol, and low estradiol+P₄ (all estradiol; n=12) versus vessels from the other non–estradiol-treatment groups (control, placebo, and P₄; n=11). Arteries from rats receiving estradiol exhibited hydroperoxide levels that were 30% lower than those observed in all other treatment groups. *P<0.01.

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 (H₂O₂). 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 P₄ 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, 0.231±0.05; low estradiol plus P₄, 0.234±0.04; control, 0.235±0.04; low estradiol, 0.246±0.01; placebo, 0.25±0.04; and P₄, 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, and both of the low-estradiol groups (low estradiol and low estradiol+P4) obtained plasma estradiol levels that are seen in intact, cycling rats.

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 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.

Resko and others have described a direct relationship between increased stress and adrenocorticotropic hormone levels with increased levels of adrenal P4 production in rats. Therefore, the acute elevation in plasma P4 and the lack of parity between P4 supplementation and plasma P4 levels do not accurately represent the actual 6-month hormone treatment. More important, this phenomenon does not alter levels of plasma estradiol and so does not compromise the reliability of these hormone measurements.

We chose pentosidine as a marker of glycoxidative damage in these studies because it has been fully structurally characterized and is a specific marker of extracellular matrix glycation. Pentosidine is regarded as 1 of the few valid in vivo biomarkers of glycoxidative damage to date. In support of our hypothesis, we found that chronic estradiol supplementation, whether administered alone or in the presence of P4, decreased levels of pentosidine in the artery wall. Increased levels of glycoxidative products have been linked to increased permeability both in vivo and in vitro. Interestingly, we observed decreased endothelial permeability to dextran (76 000 molecular weight) in isolated, perfused, carotid arteries from these estradiol-implanted rats (P<0.01; B.A.W. et al, unpublished data, 1998) as well as diminished basal rates of LDL accumulation (P<0.01). It is possible that estradiol-mediated decreases in arterial glycoxidative products prevent LDL accumulation by decreasing endothelial permeability and/or binding of LDL to the artery wall.

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). Specifically, estradiol increases activity and decreases food intake, 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 and increasing the sympathetic nervous system activity to white adipose tissue. 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. This phenomenon is seen after menopause in women and after ovariectomy in rats. 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 as well as decrease hepatic glucose production. Furthermore, ovariectomy in rats results in decreased glucose uptake as well as diminished glycogen synthesis in muscles, 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. 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-treated rats were due to an antioxidant effect of estradiol on the artery wall or secondary to decreased glycemic stress. Estradiol has been shown to confer direct antioxidant protection against oxygen radicals and to alter levels of antioxidant enzymes. 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-
ative 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 (H₂O₂) and protein modification. Later, this same group 59 described the generation of H₂O₂ 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 P₄-implanted rats exhibited a 1.3-fold increase in H₂O₂, 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-κB. 52,53 Clearly, this combination of oxidative and glucemic 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 with estrogens, as was seen in our study. Estrogen therapy also has been observed to blunt the increase in body weight and attenuate the change in body fat to a central (android) distribution in early-postmenopausal women as well. 54 This result is similar to what we observed in our estradiol-implanted rats. However, Wagner et al 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, 32 vascular stiffening, 56 and LDL binding, 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

This research was supported by a grant from the National Institutes of Health’s Heart, Lung, and Blood Institute, Bethesda, Md (RO1 HL 55667) (to J.C.R.).

References

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doi: 10.1161/01.ATV.19.4.840

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