Estrogen has opposing effects on vascular reactivity in obese, insulin-resistant male Zucker rats

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First published January 11, 2002; 10.1152/japplphysiol.00559.2001.—We hypothesized that estradiol treatment would improve vascular dysfunction commonly associated with obesity, hyperlipidemia, and insulin resistance. A sham operation or 17β-estradiol pellet implantation was performed in male lean and obese Zucker rats. Maximal vasorelaxation (VC) to phenylephrine (PE) and potassium chloride was exaggerated in control obese rats compared with lean rats, but estradiol significantly attenuated VC in the obese rats. Estradiol reduced the PE EC50 in all groups. This effect was cyclooxygenase independent, because preincubation with indomethacin reduced VC response to PE similarly in a subset of control and estrogen-treated lean rats. Endothelium-independent vasodilation (VD) to sodium nitroprusside was similar among groups, but endothelium-dependent VD to ACh was significantly impaired in obese compared with lean rats. Estradiol improved VD in lean and obese rats by decreasing EC50 but impaired function by decreasing maximal VD. The shift in EC50 corresponded to an upregulation in nitric oxide synthase III protein expression in the aorta of the estrogen-treated obese rats. In summary, estrogen treatment improves vascular function in male insulin-resistant, obese rats, partially via an upregulation of nitric oxide synthase III protein expression. These effects are counteracted by adverse factors, such as hyperlipidemia and, potentially, a release of an endothelium-derived contractile agent.

hormones; non-insulin-dependent diabetes mellitus; nitric oxide synthase; indomethacin; cyclooxygenase agents, attenuating contraction to certain agents (7, 36) and potentiating contraction to others (10, 28). These findings are attributed to the ability of estrogen to influence nitric oxide (NO) availability, cyclooxygenase products, calcium handling, free radical production, and lipid profile (9). Despite the plethora of evidence supporting a cardioprotective effect by estrogen, findings from the recent Heart and Estrogen/progestin Replacement Study, a prospective, randomized, placebo-controlled clinical trial, indicate that hormone replacement therapy (HRT), estrogen plus progestins, is not protective for secondary prevention of cardiovascular disease until after 2–3 yr of use (15). In fact, the use of HRT actually increased risk for cardiovascular events in the first year of treatment while increasing risk for venous thromboembolism by threefold (12, 35). Similarly, in a recent case-control study of postmenopausal estrogen use and risk of incident myocardial infarction in diabetic women enrolled at Group Health Cooperative of Puget Sound (19), multivariate-adjusted relative risk for myocardial infarction was 1.18 for present HRT users of <2 yr, 0.69 for present HRT users of 2–6 yr, and 0.18 for HRT users of >6 yr. Although this study was limited by the small number of subjects and low prevalence of HRT use, it corresponds to findings from the recent Heart and Estrogen/progestin Replacement Study (15). Therefore, the role of estrogen in cardiovascular disease prevention is unclear at this time.

There is a paucity of information regarding the influence of HRT on heart disease and cardiovascular function in postmenopausal women with non-insulin-dependent diabetes mellitus (NIDDM). Estrogen improves insulin resistance and lipoprotein profile in postmenopausal women with NIDDM (4); therefore, it might also improve vascular function in this cohort of women. The prevalence of NIDDM is ~15–30% in the population of individuals aged 55 yr or older in the United States, a population that is expected to grow ~20–30% over the next 50 yr, according to recent estimates by the US Census Bureau. Epidemiological studies have reported that the presence of diabetes
increases the incidence of cardiovascular disease in both men and women (3, 24), results in widespread microvascular (21) and macrovascular complications (25), and increases risk for stroke (27). Furthermore, obesity, hypertension, hypercholesterolemia, and dyslipidemia represent independent risk factors for cardiovascular disease progression. Although partially explained by the increased survival rate in women than men without diabetes, the presence of diabetes removes the normal gender difference in the prevalence of coronary heart disease (2). Therefore, questions include the following. 1) Is estrogen vascular protective in the setting of insulin resistance and glucose intolerance? 2) If not, what mechanism(s) or factor(s) prevents cardioprotection by estrogen in the presence of insulin-resistance? In the present study, we hypothesized that estrogen administration might protect against vascular complications in an obese male rat model of NIDDM, the fatty Zucker rat. The male rat was chosen to study the estrogen interaction with insulin resistance, because the male obese rats show a higher incidence of glucose intolerance than the female counterparts (16). Our primary focus was on the contributing role of a cyclooxygenase-dependent factor(s) and NO as potential mechanisms in contributing to changes in vascular function. Our hypotheses were that 1) estradiol administration would improve endothelium-dependent vasodilation to ACh via an upregulation of NO synthase (NOS) III protein expression with a subsequent increase in NO availability, and 2) estradiol administration would attenuate the exaggerated vasoconstrictor responses commonly observed in humans and rodent models of obesity, dyslipidemia, and insulin resistance.

METHODS

Animals

A total of 22 male obese and 32 lean Zucker rats (Charles River Laboratories) at ~10–14 wk of age were used for the present study. The obese Zucker rat is a commonly used rat model for NIDDM, showing similar characteristics as human NIDDM obesity, hyperinsulinemia, dyslipidemia, insulin resistance, and moderate hypertension (23).

Rats underwent estradiol pellet implantation or sham operation under halothane anesthesia (1.0–1.5%). In one-half of the lean and obese Zucker rats, a 21-day 17β-estradiol (0.1-mg) pellet (Innovative Research) was implanted subcutaneously. The control rats underwent a sham operation. The rats were housed separately and fed standard rat chow and water ad libitum. Food consumption was monitored from the time of operation until the in vitro bioassay.

Aortic Ring Preparation and Vascular Tension Recordings

Two to three weeks after pellet or sham operation, control and estrogen-treated rats were weighed and then euthanized by an overdose of halothane inhalation. A midsternal split was quickly performed, and the descending thoracic aorta was carefully excised and placed in ice-cold Krebs buffer (concentrations in mM: 118.3 NaCl, 4.7 KCl, 1.6 CaCl2, 1.2 KH2PO4, 25 NaHCO3, 1.2 MgSO4, and 11.1 dextrose (Sigma Chemical)). The aorta was cleaned of excess fat, and the aortic rings (1.5–2.0 mm) were placed in oxygenated chambers (95% O2-5% CO2) superfused with Krebs buffer solution and maintained at 37°C, pH 7.4. Four to eight rings were used from each rat for experiments 1 and 2 (described below). A single ring from each rat was suspended between two wire stirrups in one of the 25-ml organ chambers of the eight-chamber myograph system. One stirrup was connected to a three-dimensional micromanipulator and the other to a force transducer.

All of the rings were stretched to 3,000 mg in 500-mg increments over a 1-h period to optimize the contractile response to potassium chloride (KCl). One dosage of KCl (60 mM) was administered to verify vascular smooth-muscle viability.

Experiment 1. Twenty-two obese and twenty-five lean rats were used for these experiments. One to four rings from each rat were exposed to a given vasoactive agent. After three washes, cumulative dose-response curves for phenylephrine (PE) (10−9–10−5 M) were obtained by administering the drug in one-half log doses. Endothelium-dependent and -independent vasodilation was determined by generating dose-response curves to ACh and sodium nitroprusside (SNP), respectively. Vasorelaxation evoked by ACh and SNP was expressed as percent relaxation determined by the percent-age of inhibition to the preconstricted tension evoked with the ECl/a to PE (range from 10−7 to 10−6 M).

Experiment 2. In a subset of each group of rats (n = 5 for each subset), two to four rings from each rat were incubated for 30 min with 10−5 M Nω-nitro-L-arginine methyl ester (L-NAME) hydrochloride, a NOS III inhibitor, and two to four rings from the same rat were maintained without L-NAME (control set of rings) in separate chambers before generation of PE, ACh, and SNP dose-response curves, as described in experiment 1. Although L-NAME has muscarinic receptor antagonistic properties, the concentration of L-NAME (10−5 M) used in these experiments should not significantly (<10%) block muscarinic receptors and is approximately an order of magnitude less than the L-NAME concentration reported to competitively displace radioligand binding of muscarinic receptors by 50% (5).

Experiment 3. In a separate experiment, three lean control (LC) and four lean, estrogen-treated (LE) rats were used to investigate the contribution of cyclooxygenase-dependent factors on vascular reactivity. Three rings from each rat were incubated for 20 min with 10−4 M indomethacin (Indo), a cyclooxygenase inhibitor, and two rings without Indo (control set of rings) were maintained in separate chambers before testing of smooth muscle viability with one dose of 60 mM KCl followed by generation of a PE (10−9–10−6M) dose-response curve. Hormonal and lipid measurements were not performed for this experiment.

Blood Chemistry and Hormonal Analysis

Blood samples were collected by a cardiac puncture in a heparinized vacutainer. Samples were placed on ice, centrifuged at 2,500 rpm for 20 min to obtain plasma fractions, stored at ~70°C, and later assayed for circulating 17β-estradiol, progesterone, and insulin concentrations (Table 1). Insulin (Linco Research, St. Charles, MO), 17β-estradiol, and progesterone (Diagnostic Products, Los Angeles, CA) were analyzed by an 125I double-antibody radioimmunoasssay. Remaining plasma samples were assayed within 1 wk for plasma glucose, total cholesterol, and triglycerides by using enzymatic determination kits purchased from Sigma Chemical.
**NOS III Protein Expression**

Eight to ten aortic rings (2–3 mm) per rat were snap frozen and stored at −80°C. On a separate day, rings were homogenized in ice-cold lysis buffer [100 mM NaCl, 50 mM Tris, pH 7.6, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholic acid, 1% Triton X-100, 1 μM phenylmethylsulfonyl fluoride, 50 μM NaF, 0.5 μM sodium orthovanadate, 10 μM leupeptin, and 20 μM aprotinin (Sigma Chemical)], incubated for 30 min at room temperature, and centrifuged at 4°C for 30 min at 15,000 rpm. A bicinchoninic acid protein assay (Pierce, Rockford, IL) was performed on the soluble fraction (supernatant). Protein samples (10 μg) were fractionated by SDS-PAGE on 8–16% wide-range gels (Novex, San Diego, CA). The proteins were then transferred to a polyvinylidene difluoride membrane (Novex) and blocked for 60 min at room temperature or overnight at 4°C with PBS blocking buffer [600 ml PBS, 0.1% Tween 20 (Bio-Rad), and 2.4 g I-Block (Bio-Rad)]. The next day, membranes were immunoblotted for 1 h against mouse endothelial cell NOS (or NOS III) primary antibody (Transduction Laboratories, Lexington, KY), washed twice in PBS blocking buffer, incubated with biotin-goat anti-mouse IgG (Zymed, San Francisco, CA), washed three times with blocking buffer, incubated with conjugated streptavidin with alkaline phosphatase for 30 min, and then washed three times with PBS blocking buffer. An assay buffer was prepared (250 ml double-distilled H2O, 2.4 ml diethylamine, pH 10.0, and 50 mg MgCl2) and used to wash the membrane twice for 5 min. The membrane was exposed to chemiluminescence substrate (CSPD, Tropix, Bedford, MA) for 5 min, and chemiluminescence was measured by a luminometer (Amersham) developed with a Konica medical film processor (QX-70, Konica). Densitometric analysis was performed (ImageQuant, Personal Densitometer SI, Molecular Dynamics), and band density of NOS III for LC, LE, and obese, estrogen-treated (OE) rats was expressed as a percentage of the density of the obese control (OC) rat band for each film.

**Statistical Analysis**

Data were expressed as means ± SE, and the statistical significance level was set to α = 0.05. To calculate the vascular response of each rat to a specific vasoactive agent (SNP, ACh, KCl, PE), the average vascular response of all of the rings for each rat at each concentration of a particular vasoactive agent was calculated. For determination of the mean vascular response within a group of rats, the mean of these averages was calculated and used for statistical analyses and data summaries. Vascular reactivity data were analyzed off-line with PRISM data analysis software (GraphPad). EC50 and the concentration to evoke a maximal response (E_{max}) were calculated for the individual concentration-response curves by using nonlinear logistic regression with the PRISM software. The mean value for the EC50 was reported as the negative logarithm of the molar drug concentrations. Statistical differences for EC50 and E_{max} among the four groups were determined by using two-factor (obese vs. lean and estrogen vs. no estrogen) analysis of variance. The influence of L-NAME on vascular responsiveness was analyzed by two-factor ANOVA (L-NAME vs. no L-NAME, estrogen vs. control) within each of the two groups: obese and lean. The influence of Indo on vascular responsiveness was analyzed by two-factor ANOVA (Indo vs. no Indo, estrogen vs. control) for the lean rats. Differences between groups at each dose concentration were not analyzed, because the study was not powered to investigate this number of comparisons (≥9 comparisons). Differences among groups and treatments for anthropometric data, blood hormone and chemical concentrations, and NOS III were analyzed by two-factor ANOVA with Statview statistical software. Post hoc comparisons with a Bonferroni correction were conducted for all overall significant tests. The correlation between the percent increase in NOS III expression and serum estradiol was also investigated.

**RESULTS**

**Anthropometric, Plasma Hormone, and Blood Chemistry Values**

Anthropometric and blood chemistry values are presented in Table 1 for the OC, OE, LC, and LE Zuckers. The data presented in Table 1 do not include the three LC rats and four LE rats used for experiment 3. For this separate experiment, mean ages (±SE) at the time of death for LC and LE rats were 15.0 ± 0.6 and 15.7 ± 0.3 yr, respectively. Mean weights (±SE) at the time of death for LC and LE groups were 356.8 ± 12.7 and 474.3 ± 2.2 g, respectively.

Obese Zuckers weighed significantly more than lean Zuckers before and after 2–3 wk of exposure to 17β-estradiol. Obese Zuckers were ∼1 wk younger than the lean Zuckers. Food intake was significantly greater in the obese Zuckers compared with the lean Zuckers, and estrogen treatment significantly reduced food intake in the OE group only. Although food intake was not significantly reduced in the LE group, weight gain over the 2- to 3-wk period was significantly less for both LE and OE groups.

### Table 1. Anthropometric characteristics and blood chemistry profile of experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Lean Control</th>
<th>Lean + Estrogen</th>
<th>Obese Control</th>
<th>Obese + Estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, wk</td>
<td>14.9 ± 1.6</td>
<td>15.0 ± 1.7</td>
<td>13.9 ± 1.3*</td>
<td>14.1 ± 1.4*</td>
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<tr>
<td>Preweight, g</td>
<td>324.5 ± 13.1</td>
<td>323.7 ± 13.3</td>
<td>474.1 ± 23.6*</td>
<td>478.5 ± 17.8*</td>
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<td>Postweight, g</td>
<td>349.2 ± 12.5</td>
<td>306.8 ± 13.7†</td>
<td>553.7 ± 15.1†</td>
<td>490.7 ± 17.5†</td>
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<tr>
<td>Food ingested, g</td>
<td>303.8 ± 32.5</td>
<td>270.9 ± 34.3</td>
<td>667.2 ± 31.6*</td>
<td>479.2 ± 44.0†</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>208.2 ± 14.2</td>
<td>196.8 ± 11.0</td>
<td>227.9 ± 21.9</td>
<td>187.2 ± 15.9</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>15.0 ± 2.2</td>
<td>44.7 ± 6.1†</td>
<td>21.8 ± 6.6</td>
<td>30.5 ± 6.3†</td>
</tr>
<tr>
<td>Progesterone, pg/ml</td>
<td>4.1 ± 0.6</td>
<td>5.5 ± 0.7</td>
<td>4.2 ± 0.4†</td>
<td>5.7 ± 0.9†</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>68.4 ± 15.0</td>
<td>112.1 ± 17.6†</td>
<td>204.7 ± 37.6*</td>
<td>307.0 ± 40.6†</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>86.7 ± 11.0</td>
<td>88.2 ± 9.4</td>
<td>148.9 ± 19.5*</td>
<td>194.8 ± 36.7*</td>
</tr>
<tr>
<td>Insulin, mg/dl</td>
<td>2.7 ± 0.6</td>
<td>1.8 ± 0.5</td>
<td>39.6 ± 13.2*</td>
<td>16.4 ± 6.3†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Significant differences: * obese vs. lean Zuckers and † estradiol vs. control, P < 0.05.
After estrogen administration, plasma estradiol concentrations increased to values typically observed in female rats. A nearly significant interaction (P = 0.0648) between drug treatment (estradiol vs. control) and group (lean vs. obese) indicated that subcutaneous estradiol administration was more effective in raising serum estradiol concentration in the lean than in the obese Zuckers.

Insulin resistance in the OC and OE groups was characterized by marked elevations in plasma insulin concentrations compared with that in the LC and LE groups (P = 0.0011). Nonfasting plasma glucose concentrations were not significantly different between lean and obese Zuckers, and estrogen treatment did not affect plasma glucose. Obesity significantly raised plasma triglyceride and total cholesterol concentrations. Estrogen did not affect total cholesterol concentrations, but estrogen significantly increased triglyceride concentrations in both LE and OE groups relative to controls (LC and OC groups).

**Vascular Response to KCl**

Maximal tension development in response to a 60 mM bolus dose of KCl in LC and LE groups was significantly lower compared with that in the OC group (Fig. 1). Estrogen treatment decreased the maximal contractile response to KCl for both the obese and lean Zuckers; however, this reduction was only significant for the OE group (P < 0.001).

**Vascular Response to PE**

As presented in Fig. 2 and Table 2, Emax in response to PE in the OC group (2,162 ± 101 mg) was significantly greater compared with that in the LC (1,827 ± 96 mg) and LE (1,855 ± 67 mg) groups. Similar to KCl findings, chronic estradiol exposure significantly reduced Emax in the OE group, such that Emax for the OE group (1,811 ± 53 mg) was similar to that of LC and LE groups.

The PE EC50 was significantly lower in the obese Zuckers (EC50 = −7.35 ± 0.11 and −7.51 ± 0.07 M for OC and OE groups, respectively) compared with the lean Zuckers (EC50 = −7.13 ± 0.11 and −7.34 ± 0.08 M for LC and LE groups, respectively). Estrogen enhanced the contractile response to PE in both the lean and obese Zuckers by significantly shifting EC50 to a lower concentration.

Incubation of the aortic rings for 15 min with L-NAME, a NOS inhibitor, significantly increased Emax and reduced EC50 in response to PE for all four groups (Fig. 3). Preincubation of L-NAME before generation of PE dose-response curves resulted in quantitatively similar shifts in both Emax and EC50 for all four groups.

When aortic rings from LC (n = 3) and LE (n = 4) groups were tested separately in experiment 3, maximal contraction to PE without Indo was similar between the two groups (Emax = 1,908 ± 217 and 2,076 ± 124 mg for LC and LE groups, respectively) (Fig. 4). Preincubation with Indo significantly decreased maximal contraction in both groups by a similar extent (Emax = 720 ± 108 and 1,100 ± 61 mg for LC and LE groups, respectively). Similarly, Indo treatment significantly shifted the EC50 for both LC and LE rats to a higher concentration, thereby reducing vascular tension for a given dose of PE. Although EC50 for the LE group of rats was lower than that for the LC group, this difference between LC and LE groups was not significant, as was observed in experiments 2 and 3.

**Vascular Response to SNP**

Percentage of relaxation to SNP was similar among the four groups of rats. Emax values for LC, LE, OC, and OE groups were 103.0 ± 2.1, 102.5 ± 2.5, 100.7 ± 1.9, and 102.4 ± 3.1%, respectively. EC50 values [−log (SNP, M)] for LC, LE, OC, and OE groups were 8.34 ± 0.17, 8.27 ± 0.15, 8.49 ± 0.14, and 8.28 ± 0.21 M, respectively. In no case did estrogen treatment and/or
the presence of the cardiovascular risk factors affect vasodilator responses to SNP. In contrast to previously reported findings (29), the preincubation and presence of L-NAME did not alter vascular responses to SNP in any group.

**Vascular Response to ACh**

In general, endothelium-dependent relaxation to ACh was impaired in the obese control rats compared with their lean counterparts. As presented in Figs. 5 and 6, E\textsubscript{max} for the OC (82.30 ± 4.82 mg) group was significantly lower compared with that for the LC (91.29 ± 1.97 mg) group. The EC\textsubscript{50} for the OC (–7.16 ± 0.13) group was significantly higher than that for the LC (–7.42 ± 0.06) group. Estrogen treatment significantly reduced the maximal capacity of the aortic rings of the OE (78.78 ± 3.27 mg) and LE (84.32 ± 2.52 mg) groups to dilate in response to ACh. However, estrogen

<table>
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<tr>
<th>Group</th>
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<th>Lean + Estrogen</th>
<th>Obese Control</th>
<th>Obese + Estrogen</th>
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<tbody>
<tr>
<td>No L-NAME, n</td>
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<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>E\textsubscript{max}, mg</td>
<td>1,827 ± 96</td>
<td>1,855 ± 67</td>
<td>2,162 ± 101§</td>
<td>1,811 ± 53†</td>
</tr>
<tr>
<td>–log EC\textsubscript{50}, M</td>
<td>7.13 ± 0.11</td>
<td>7.34 ± 0.08†</td>
<td>7.35 ± 0.11*</td>
<td>7.51 ± 0.07†</td>
</tr>
<tr>
<td>L-NAME, n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>E\textsubscript{max}, mg</td>
<td>2.348 ± 185‡</td>
<td>2.513 ± 97‡</td>
<td>2.698 ± 85§‡</td>
<td>2.435 ± 94‡‡</td>
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<tr>
<td>–log EC\textsubscript{50}, M</td>
<td>7.22 ± 0.17‡</td>
<td>7.57 ± 0.10‡†</td>
<td>7.58 ± 0.08‡*</td>
<td>7.70 ± 0.11‡‡</td>
</tr>
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</table>

Values are means ± SE; n, no. of animals. L-NAME, N\textsuperscript{n}-nitro-L-arginine methyl ester; E\textsubscript{max}, concentration to evoke a maximal response.

*Significantly different from lean counterparts; †significant estradiol effect; §L-NAME vs. no L-NAME; ‡obese estrogen-treated group vs. obese control, lean estrogen-treated, and lean control groups: P < 0.05.

Fig. 3. Pretreatment of the aortic rings with N\textsuperscript{n}-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) III inhibitor, resulted in a significant increase in E\textsubscript{max} and decrease in EC\textsubscript{50} in both lean (A) and obese (B) Zucker rats. Values are means ± SE.

Fig. 4. Preincubation with 10\textsuperscript{-4} M indomethacin (Indo), a cyclooxygenase enzyme inhibitor, significantly and similarly reduced E\textsubscript{max} (A) and increased EC\textsubscript{50} (B) in lean Zucker rats. Values are means ± SE. *Significantly different from no Indo group, P < 0.05.
treatment enhanced ACh-mediated vasodilation by significantly shifting EC\textsubscript{50} in LE (−7.60 ± 0.09) and OE (−7.40 ± 0.11) groups to a lower drug concentration. Vasorelaxation to ACh was abolished in the presence of L-NAME (data not presented), indicating that the ACh-mediated vasodilation in the aorta of the male Zucker rats was primarily dependent on NOS activity.

Protein Quantification

NOS III protein expression was significantly greater (199.3 ± 32.9\%) in the LC group compared with the OC group (Figs. 7 and 8). Estrogen treatment significantly increased (191.4 ± 19.7\%) expression of NOS III from aortic rings in the OE group but not in the LE group (Fig. 7). Representative bands from each of the four groups are presented in Fig. 8. Serum estradiol concentration was not significantly correlated to the percent increase in NOS III expression (\(P = 0.99, R = 0.001\)).

DISCUSSION

To our knowledge, this is the first study to assess the influence of chronic estradiol on vascular function in an obese, insulin-resistant rat model. Our original hypotheses were the following: 1) chronic estradiol administration would improve endothelium-dependent vasodilation to ACh via an upregulation of NOS III protein expression with a subsequent increase in NO availability, and 2) estradiol administration would attenuate the exaggerated vasoconstrictor responses commonly observed in humans and rodent models of obesity, dyslipidemia, and insulin resistance. Our hypotheses were partially confirmed. As hypothesized, 2–3 wk of estrogen treatment partially restored ACh-mediated vasodilation in the obese Zucker rats by increasing the vasodilatory responsiveness to ACh. Estrogen also reduced the exaggerated contractile responses to KCl and PE in the obese Zucker rats. Contrary to our hypotheses, estrogen reduced the maximal vasodilatory capacity to ACh and enhanced contractile responses to PE by reducing the PE EC\textsubscript{50} in both lean and obese Zucker rats. This shift in EC\textsubscript{50} by estrogen does not appear to occur via an upregulation or release of a cyclooxygenase-dependent factor, because preincubation of aortic rings with Indo before generation of PE dose-response curves similarly affected vascular responses in both LC and LE groups of rats.

Vasoconstrictor Effects

Similar to previous findings (32), the maximal contractile responses to KCl and PE were exaggerated in the OC group compared with the LC group. This effect has been attributed to increased activation of voltage-
NO activation. This shift in EC_{50} was not significant for the lean rats in experiment 3, but the lack of significance may be attributable to the small number of animals used for this separate experiment. The presence of estrogen or a substance upregulated by estrogen apparently enhanced the vascular reactivity to PE in the male Zucker rats. These findings indicate that estrogen is sensitizing the early responsiveness of the vasculature to PE and, at the same time, limiting the maximal contractile capacity of the vessel. The potential contribution of a cyclooxygenase-dependent product from the endothelium by estrogen was investigated in a group of lean Zucker rats only. We did not find that estrogen increased the availability of a cyclooxygenase-dependent factor to contribute to the exaggerated sensitivity to PE. In fact, the preincubation of 10^{-4} M Indomethacin evoked similar changes in both E_{max} and EC_{50} in both LC and LE groups of rats (Fig. 4). A second factor that may contribute to the exaggerated vascular responses in the OE and LE groups is the elevated triglyceride concentrations after estrogen treatment. Similar to previous findings in humans and animals, estrogen treatment increased triglyceride concentrations in the male lean and obese Zucker rats. The hyperlipidemia after estrogen administration worsens glomerular injury in the Zucker rats by increasing low-density, triglyceride-rich lipoproteins and albuminuria, despite reduced food intake and weight gain (34). Elevated circulating triglyceride-rich lipoproteins may have similar effects on other vascular beds. In a recently published randomized, placebo-controlled study (20) in 20 postmenopausal women with NIDDM, 0.625 mg/day of conjugated equine estrogen for 8 wk did not significantly improve endothelium-dependent dilation of the brachial artery after reactive hyperemia compared with placebo treatment. The failure by estrogen to improve endothelium-dependent vasodilation corresponded to a significant increase (~16%) in circulating triglyceride concentrations.

**Vasodilator Effects**

Before estrogen administration, endothelium-dependent vasodilation to ACh was significantly impaired in obese Zuckers compared with the lean Zuckers. E_{max} was reduced and EC_{50} was significantly increased in the OC group compared with the LC group (Figs. 5 and 6). Impaired endothelium-dependent relaxation is a common manifestation of obesity, hypercholesterolemia, hyperlipidemia, insulin resistance, and estrogen deficiency. Endothelial dysfunction may result from reduced NO bioavailability, decreased endothelium-dependent Ca^{2+} channels (32), impaired ability of insulin to attenuate contractile responses to pressor agents (39), and increased oxidant stress (38) in the fatty Zucker rat and rodent models of obesity and diabetes. As hypothesized, estrogen reduced the maximal contractile responses to 10^{-6} M KCl and PE in the OE group. It is unlikely that this effect was due to the upregulation of NOS III or a greater constitutive release of NO in the OE group, because preincubation of L-NAME before generation of these dose-response curves resulted in quantitatively similar shifts in both E_{max} and EC_{50} for OC and OE groups. Estrogen likely reduced the maximal pressor response to KCl and PE in the OE group by modulating a NOS-independent cellular mechanism, such as calcium handling (40), free radical production (1), protein kinase C (PKC) activity (18), cyclooxygenase product availability, or sensitivity of the vasculature to insulin receptor activation. There are several impaired cellular pathways in the insulin-resistant obese male Zuckers that may be modified by estrogen treatment. Lipid peroxidation is elevated in the fatty, insulin-resistant Zucker rat (26), whereas estradiol reduces lipid peroxidation via inhibition of superoxide radical generation and chain propagation (1). A second possibility is that vascular PKC activity may be increased in the obese Zucker rats (22), resulting in an exaggerated maximal contractile response to PE. In contrast, estrogen could potentially reduce PKC-mediated aortic contraction (18), thereby counteracting the abnormal response in the fatty Zucker rat.

Contrary to its influence on E_{max}, estrogen enhanced the exaggerated contractile response to PE in both lean and obese Zucker rats by significantly reducing EC_{50}. Unlike E_{max}, estrogen treatment similarly affected this surrogate marker of vascular reactivity in both the lean and obese Zucker rats and was independent of

**Fig. 8.** Western blot analysis and SDS-PAGE. Representative bands are shown for NOS III (140-kDa protein) protein expression for LC, LE, OC, and OE groups of Zucker rats.

**Fig. 7.** Endothelial-derived nitric oxide synthase (endothelial cell NOS or NOS III) protein expression was quantified by Western blot analysis. NOS III protein expression was significantly lower in the OC group compared with LC and LE groups (**P < 0.05**). Estrogen treatment significantly increased NOS III protein expression in the OE group (**P < 0.05**) such that protein expression for the OE group was similar to that for LC and LE groups of Zucker rats. Values are means ± SE.
derived relaxing factors other than NO (e.g., endothelium-derived hyperpolarizing factor, prostacyclin), increased endothelium-derived contractile factors (e.g., endothelin, thromboxane A₂, PGH₂), increased superoxide production, or a combination of these mechanisms. Because L-NAME completely blocked vasodilation in response to ACh in all groups, the ACh-mediated vasorelaxation in the aortic rings from our population of animals occurred via NO rather than a cyclooxygenase-derived relaxing factor (prostaglandin) or endothelium-derived hyperpolarizing factor. The lack of a difference in vasorelaxation to SNP between LC and OC groups indicates that an impaired sensitivity of the vessel to NO could not explain the attenuated ACh-mediated relaxation in the OC group. These findings, along with the reduced expression of NOS III in the aortic rings of the OC group, indicate that ACh-induced NO release was impaired in the OC group. Additionally, decreased bioavailability of NO associated with increased oxidative stress may contribute to the endothelial dysfunction associated with diabetes (26).

Two to three weeks of estradiol treatment partially restored the impaired ACh-mediated vasodilation in the obese Zucker rats by significantly reducing EC₅₀ in the OE group. NOS III protein expression was significantly lower in the OC group compared with the LC group, but estrogen administration profoundly increased expression of NOS III in the aortic rings of the OE group. These findings are consistent with the estrogen-mediated leftward shift in EC₅₀ of the ACh curves in the OE group. Estrogen treatment also shifted the ACh curve leftward in the LE group via a reduction in EC₅₀, despite a lack of an effect by estrogen on NOS III protein expression. These findings suggest that the improved endothelium-dependent vasodilation in response to ACh in the LE and OE groups occurred via a different mechanism. One explanation may be strain differences in estrogen receptor density or responsiveness. In hypothalamic preoptic areas of the brain, the density of estrogen receptors in obese Zucker rats is less compared with that in lean Zucker rats, resulting in less sexual receptivity in the obese rats (31). Sexual behavior in the obese Zucker rats after estrogen treatment is also impaired, indicating a reduced receptor responsiveness in this group of animals (8). Other possible mechanisms to explain the differential response to estrogen treatment in lean and obese rats include the following: 1) a relatively greater improvement in circulating insulin concentrations in the obese rats, and 2) strain variations in intracellular signaling. Estrogen may enhance the expression of NOS III (a genomic-based mechanism for enhanced endothelial function) or increase NOS III activity rapidly by a nongenomic mechanism (6). This enhanced NOS III activity does not involve increases in endothelial cytosolic Ca²⁺ (6). Recently, Haynes et al. (13) demonstrated that membrane estrogen receptor activation increased NOS III activity through a phosphatidylinositol 3-kinase-Akt-dependent pathway, resulting in phosphorylation of NOS III and enhanced activity. Although we did not interrogate this mechanism, it is possible that it may contribute to the leftward shift in the ACh-mediated curve in the LE group.

In contrast to our original hypothesis and the aforementioned beneficial effects by estrogen on endothelium-dependent vasodilation, maximal relaxation to estrogen was reduced after estrogen treatment in both lean and obese male Zuckers. This finding was unexpected but could be explained by increased triglycerides that may impair ACh-induced vasodilation and limit the maximal dilatory capacity of the vessel. The ACh-mediated vasodilation may be confounded by the interaction between estrogen and PE. In a study by Vedernikov and colleagues (37), estradiol treatment significantly increased ACh-induced relaxation in aortic rings precontracted with KCl but not in rings precontracted with PE. Therefore, although estradiol improves ACh-mediated vasodilation via a shift in EC₅₀, its effect on maximal relaxation is attenuated in the presence of PE in the male Zucker rats.

In contrast to previously reported findings (29), the preincubation and presence of L-NAME did not alter vascular responses to SNP in any group. Removal of endogenous sources of NO (e.g., endothelium removal or NOS inhibition) has consistently been shown to reduce the vascular smooth muscle cell sensitivity to exogenous sources of NO. It is not clear why we did not detect similar differences, but we speculate that additional rats may be needed to detect small differences or shifts in vascular responsiveness.

Limitations

Confounding factors in the interpretation of the results of the present study include the following: 1) sex differences in responsiveness to estrogen treatment, 2) higher baseline estrogen concentrations in the OC group compared with the LC group, 3) the change in estradiol concentrations after estrogen treatment, 4) the presence of testosterone in the control Zuckers, 5) strain differences among rat models, and 6) distinguishing between the influence of estrogen and the weight loss associated with estrogen administration.

In summary, estrogen had opposing effects on vascular reactivity in the male insulin-resistant obese Zucker rat. Estrogen ameliorated vascular dysfunction in the obese, insulin-resistant male Zuckers by improving the vasodilatory responsiveness to ACh and decreasing the exaggerated maximal contractile responses to KCl and PE. These effects by estrogen likely occurred via NO-dependent and -independent mechanisms. These effects were not observed in the lean Zuckers, indicating that estrogen interacts with signaling pathways that are dysregulated in the obese Zucker but not in the lean Zucker.

Estrogen adversely influenced vascular function in both lean and obese groups of Zuckers. Estrogen treatment reduced the drug concentration required to achieve 50% of the maximal pressor response to PE.
This effect was NO independent, because the same relationship existed with the pretreatment of L-NAME before generation of the PE curve. Furthermore, this finding was not a result of a cyclooxygenase-dependent factor, because preincubation of the rings with Indo affected vascular responses to PE similarly in both the LC and LE groups of rats. Therefore, the mechanism by which estrogen imposes these adverse effects is unclear but may be a result of vascular damage resulting from increased circulating triglyceride concentrations. This factor poses a risk for vasospasm and might explain the increased risk of a cardiovascular event in the first year after initiation of HRT in postmenopausal women with underlying heart disease.

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