Estrogen has Opposing Effects on Vascular Reactivity in
Obese, Insulin-Resistant Male Zucker Rats

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Abbreviated Title: Estrogen affects vascular reactivity in male Zucker rats

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ABSTRACT

The influence of chronic estradiol treatment on vascular function in obese, insulin-resistant male Zucker rats was assessed. We hypothesized that estradiol administration would improve endothelium-dependent vasodilation and attenuate vasoconstrictor responses commonly observed in rodent models of obesity, hyperlipidemia, and insulin-resistance via an upregulation of NOSIII protein expression. A sham operation or a 21-day release 17β-estradiol (0.1 mg) pellet implantation was performed in male lean and obese Zucker rats. Maximal contractile responses to phenylephrine (PE) and potassium chloride (KCl) were exaggerated in the untreated obese Zucker rats compared to the lean Zucker rats, but estrogen treatment significantly attenuated this response in the obese Zucker rats. In contrast, estradiol reduced the PE concentration required to evoke 50% of the maximal tension (EC$_{50}$) in lean and obese rats. This effect was not a result of a cyclooxygenase-dependent factor, because preincubation of the rings with $10^{-4}$M indomethacin similarly reduced the contractile response to PE in a subset of LC and LE rats. Endothelium-dependent (acetylcholine, ACh) and -independent vasodilation (sodium nitroprusside, SNP) was determined following precontraction of the aorta with PE ($\approx 10^{-6}$M). Vasorelaxation to SNP was similar among all groups, but vasorelaxation to ACh was significantly impaired in the obese Zucker rats compared to the lean Zucker rats. Estradiol improved vasorelaxation in lean and obese Zucker rats by decreasing EC$_{50}$, but impaired function by decreasing maximal vasorelaxation. The shift in EC$_{50}$ corresponded to an upregulation in NOSIII protein expression in the aorta of the estrogen-treated obese group. In conclusion, estrogen treatment improves vascular reactivity in male insulin-resistant, obese Zucker rats partially via an upregulation of NOSIII protein expression. However, these effects are
counteracted by adverse factors, such as hyperlipidemia and potentially a release of an endothelium-derived contractile agent contributing to exaggerated vascular sensitivity to PE.

**Keywords:** hormones, NIDDM, nitric oxide synthase, indomethacin, cyclooxygenase
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>EC$_{50}$</td>
<td>effective concentration for 50% of the maximal response</td>
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<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
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<td>$E_{max}$</td>
<td>concentration to evoke a maximal response</td>
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<tr>
<td>ERT</td>
<td>estrogen replacement therapy</td>
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<tr>
<td>HERS</td>
<td>Heart and Estrogen/progestin Replacement Study</td>
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<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
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<tr>
<td>INDO</td>
<td>indomethacin</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
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<tr>
<td>LC</td>
<td>lean control</td>
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<tr>
<td>LE</td>
<td>lean plus estrogen treatment</td>
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<tr>
<td>L-NAME</td>
<td>Nω-Nitro-L-Arginine Methyl Ester Hydrochloride</td>
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<tr>
<td>NIDDM</td>
<td>non insulin-dependent diabetes mellitus</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>OC</td>
<td>obese control</td>
</tr>
<tr>
<td>OE</td>
<td>obese plus estrogen treatment</td>
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<tr>
<td>PE</td>
<td>phenylephrine</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>SNP</td>
<td>sodium nitroprusside</td>
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INTRODUCTION

In both healthy postmenopausal women (33) and those with underlying heart disease (14), findings from observational studies performed in the last three decades indicate that estrogen replacement therapy (ERT) reduces the rate of cardiovascular events. In male-to-female transsexuals, ERT improves endothelium-dependent, flow-mediated vasodilation (30). In support of these clinical studies, acute and chronic estradiol administration causes both endothelium-dependent (11) and -independent vasodilation (11, 17). Estrogen has mixed effects on vascular reactivity to contractile agents, attenuating contraction to certain agents (7, 36) and potentiating contraction to others (10, 28). These findings are attributed 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 (HERS), a prospective, randomized, placebo-controlled clinical trial, indicate that hormone replacement therapy (HRT), both estrogen plus progestins, is not protective for secondary prevention of cardiovascular disease until after 2 to 3 years 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 three-fold (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 current users of HRT of less than 2 years, 0.69 for current users of HRT for 2 to 6 years, and 0.18 for users of HRT greater than 6 years. Although this study was limited by the small number of subjects and low prevalence of HRT use, it
corresponds to findings from the recent HERS 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 hormone replacement therapy 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), so it might also improve vascular function in this cohort of women. The prevalence of NIDDM is approximately 15-30% in the population of individuals aged 55 years or older in the United States, a population that is expected to grow approximately 20% to 30% over the next 50 years according to recent estimates by the U.S. Census Bureau. Epidemiological studies have reported that the presence of diabetes increases the incidence of cardiovascular disease in both men and women (2, 24), results in widespread microvascular (21) and macrovascular complications (25), and increases risk for stroke (27). Furthermore, the existence of 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 sex difference in the prevalence of coronary heart disease (3). Therefore, questions include the following: (1) is estrogen vascular protective in the setting of insulin resistance and glucose intolerance? and (2) if not, what mechanism(s) or factor(s) prevent 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 nitric oxide (NO) as potential mechanisms in contributing to changes in vascular function. Our hypotheses were: (1) estradiol administration would improve endothelium-dependent vasodilation to acetylcholine (ACh) via an upregulation of NOSIII 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 twenty-two male obese and thirty-two lean Zucker rats (Charles River Laboratories, Inc.) at approximately 10 to 14 weeks of age were used for the present study. The obese Zucker rat is a commonly used rat model for non-insulin-dependent diabetes mellitus (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 half of the lean and obese Zuckers, 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 mid-sternal 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]). The aorta was cleaned of excess fat and the aortic rings (1.5 mm to 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 8-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 3000 mg in 500 mg increments over a 1-hour 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 3 washes, cumulative dose response curves for phenylephrine (PE) (10⁻⁹ to 10⁻⁵M) were obtained by administering the drug in ½ 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 “%Relaxation” determined by % of inhibition to the pre-constricted tension evoked with the effective concentration for 50% of the maximal response (EC₅₀) to PE (range from 10⁻⁷M to 10⁻⁶M).
Experiment 2

In a subset of each group of rats (n=5 for each subset), 2 to 4 rings from each rat were incubated for 30 minutes with $10^{-5}$ M Nω-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME), a nitric oxide synthase III (NOSIII) inhibitor, and 2 to 4 rings from the same rat were maintained without L-NAME (control set of rings) in separate chambers prior to 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 ($\leq 10\%$) 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, 3 lean control (LC) and 4 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 minutes with $10^{-4}$ M indomethacin (INDO), a cyclooxygenase inhibitor, and 2 rings without INDO (control set of rings) were maintained in separate chambers prior to testing smooth muscle viability with one dose of 60mM KCL followed by generation of a PE ($10^{-9}$ to $10^{-5}$ M) 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 2500 rpm for 20 minutes to obtain plasma fractions, stored at -70°C, and later assayed for circulating 17β-estradiol, progesterone, and insulin concentrations (Table 1). Insulin (Linco Research, Inc., St. Charles, MO), 17β-estradiol, and progesterone
(Diagnostic Products Corporation, Los Angeles, CA) were analyzed by an $^{125}$I double-antibody radioimmunoassay. Remaining plasma samples were assayed within one week for plasma glucose, total cholesterol, and triglycerides using enzymatic determination kits purchased from Sigma.

**NOSIII Protein Expression**

Eight to ten aortic rings (2 mm to 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 PMSF, 50 µM NaF, 0.5 µM sodium orthovanadate, 10 µM leupeptin, and 20 µM aprotinin [Sigma Chemical Co.]), incubated for 30 minutes at room temperature, and centrifuged at 4°C for 30 minutes at 1000 rpm. A BCA 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 PVDF membrane (Novex) and blocked for 60 minutes at room temperature or overnight at 4°C with PBS blocking buffer (600 ml PBS, 0.1% Tween-20 [Biorad], and 2.4g I-Block [Biorad]). The following day, membranes were immunoblotted for one hour against mouse ecNOS (or NOSIII) 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 minutes, and then washed 3 times with PBS blocking buffer. An assay buffer was prepared (250 ml ddH$_2$O, 2.4 ml of DEA, pH 10.0, and 50 mg of MgCl$_2$) and used to wash the membrane twice for 5 minutes. The membrane was exposed to Chemiluminescence substrate (CSPD, Tropix, Bedford, MA) for 5 minutes and chemiluminescence was measured on Hyperfilm ECL.
(Amersham, England) developed with a Konica Medical Film Processor (QX-70, Konica Corporation, Japan). Densitometric analysis was performed (ImageQuantNT, Personal Densitometer SI, Molecular Dynamics, Inc.), and band density of NOSIII for LC, LE, and OE was expressed a percentage of the density of the OC band for each film.

STATISTICAL ANALYSIS

Data was expressed as mean±SEM and the statistical significance level was set to α=0.05. To calculate the vascular response of each rat to a specific vasoactive factor (SNP, Ach, KCl, PE), the average vascular response of all 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 was analyzed off-line using PRISM data analysis software (GraphPad). EC$_{50}$ and concentration to evoke a maximal response (E$_{max}$) were calculated for the individual concentration-response curves using non-linear logistic regression with the PRISM software. The mean value for the EC$_{50}$ was reported as the negative logarithm of the molar drug concentrations. Statistical differences for EC$_{50}$ and E$_{max}$ among the four groups were determined 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 (approximately 9 comparisons). Differences among groups and treatments for anthropometric data, blood hormone and chemical concentrations, and
NOSIII were analyzed by two-factor ANOVA using Statview statistical software. Post hoc comparisons using a Bonferonni correction were conducted for all overall significant tests. The correlation between the percentage increase in NOSIII 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 obese control (OC), estrogen-treated obese (OE), LC, and LE Zuckers. The data presented in Table 1 does not include the 3 LC rats and 4 LE rats used for Experiment 3. For this separate experiment, mean ages (±SEM) at the time of sacrifice for LE and LC rats were 15.0±0.6 and 15.7±0.3 years, respectively. Mean weights (±SEM) at the time of sacrifice for LE and LC groups were 356.8±12.7 and 474.3±2.2 grams, respectively.

Obese Zuckers weighed significantly more than lean Zuckers before and after 2 to 3 weeks of exposure to 17β-estradiol. Obese Zuckers were approximately 1 week younger than the lean Zuckers. Food intake was significantly greater in the obese Zuckers compared to 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 week period was significantly less for both LE and OE groups.

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 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 to LC and LE groups (p=0.0011). Non-fasting 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 Potassium Chloride (KCl)**

Maximal tension development in response to a 60 mM bolus dose of KCl in LC and LE groups was significantly lower compared to the OC group (Figure 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 Phenylephrine**

As presented in Figure 2 and Table 2, $E_{\text{max}}$ in response to PE in the OC group (2162±101 mg) was significantly greater compared to LC (1827±96 mg) and LE (1855±67 mg) groups. Similar to KCl findings, chronic estradiol exposure significantly reduced $E_{\text{max}}$ in the OE group, such that $E_{\text{max}}$ for the OE group (1811±53 mg) was similar to that of LC and LE groups.

The PE concentration required to achieve 50% of the maximal contractile response was significantly lower in the obese Zuckers ($EC_{50} = -7.35±0.11 \text{ M}$ and -7.51±0.07 M for OC and OE groups, respectively) compared in the lean Zuckers ($EC_{50} = -7.13±0.11 \text{ M}$ 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 $EC_{50}$ to a lower concentration.
Incubation of the aortic rings for 15 minutes with L-NAME, a NOS inhibitor, significantly increased $E_{\text{max}}$ and reduced $EC_{50}$ in response to PE for all four groups (Figure 3, A and B). Preincubation of L-NAME prior to generation of PE dose-response curves resulted in quantitatively similar shifts in both $E_{\text{max}}$ and $EC_{50}$ 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 ($E_{\text{max}}=1908\pm217\text{mg}$ and $2076\pm124\text{mg}$ for LC and LE groups, respectively) (Figure 4). Preincubation with INDO significantly decreased maximal contraction in both groups by a similar extent ($E_{\text{max}}=720\pm108\text{mg}$ and $1100\pm61\text{mg}$ for LC and LE groups, respectively). Likewise, INDO treatment significantly shifted the $EC_{50}$ for both LC and LE rats to a higher concentration, thereby reducing vascular tension for a given dose of PE. Although $EC_{50}$ for the LE group of rats was lower than the LC group, this difference between LC and LE groups was not significant as was observed in Experiments 2 and 3.

**Vascular Response to Sodium Nitroprusside**

Percentage relaxation to SNP was similar among the four groups of rats. $E_{\text{max}}$ values for LC, LE, OC, and OE groups were $103.0\pm2.1\%$, $102.5\pm2.5\%$, $100.7\pm1.9\%$, and $102.4\pm3.1\%$, respectively. $EC_{50}$ values ($-\log[\text{SNP,M}]$) for LC, LE, OC, and OE groups were $8.34\pm0.17$, $8.27\pm0.15$, $8.49\pm0.14$, and $8.28\pm0.21\text{M}$, 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 Acetylcholine

In general, endothelium-dependent relaxation to ACh was impaired in the obese control rats compared to their lean counterparts. As presented in Figures 5 and 6, $E_{\text{max}}$ for the OC (82.30±4.82mg) group was significantly lower compared to the LC (91.29±1.97mg) group. The $EC_{50}$ for the OC (-7.16±0.13) group was significantly higher than LC (-7.42±0.06) group. Estrogen treatment significantly reduced the maximal capacity of the aortic rings of the OE (78.78±3.27mg) and LE (84.32±2.52mg) groups to dilate in response to ACh. However, estrogen treatment enhanced ACh-mediated vasodilation by significantly shifting $EC_{50}$ 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 NO synthase activity.

Protein Quantification

NOSIII protein expression was significantly greater (199.3 32.9%) in the LC group compared to the OC group (Figures 7 and 8). Estrogen treatment significantly increased (191.4 19.7%) expression of NOSIII from aortic rings in the OE group, but not in the LE group (Figure 7). Representative bands from each of the four groups are presented in Figure 8. Serum estradiol concentration was not significantly correlated to the percentage increase in NOSIII 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 NOSIII 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 to 3 weeks 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 concentration required to achieve 50% of the maximal tension in both lean and obese Zucker rats. This shift in EC$_{50}$ by estrogen does not appear to occur via an upregulation or release of a cyclooxygenase-dependent factor, because preincubation of aortic rings with INDO prior to 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 to LC group. This effect has been attributed to increased activation of voltage-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 NOSIII or a greater constitutitive release of NO in the OE group, because preincubation of L-NAME prior to 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), while 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 $E_{\text{max}}$, estrogen enhanced the exaggerated contractile response to PE in both lean and obese Zuckers by significantly reducing $EC_{50}$, the concentration of PE required to evoke 50% of the maximal contractile response. Unlike $E_{\text{max}}$, estrogen treatment similarly affected this surrogate marker of vascular reactivity in both the lean and obese Zuckers and was independent of NOS 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 $10^{-4}$ INDO evoked similar changes in both $E_{max}$ and $EC_{50}$ in both LC and LE groups of rats (Figure 4). A second factor which may contribute to the exaggerated vascular responses in the OE and LE groups is the elevated triglyceride concentrations following 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 following estrogen administration worsens glomerular injury in the Zucker rats by increasing low-density triglyceride-rich lipoproteins and albuminuria, despite reducing 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 weeks did not significantly improve endothelium-dependent dilation of the brachial artery following reactive hyperemia compared to placebo treatment. The failure by estrogen to improve endothelium-dependent vasodilation corresponded to a significant increase (~16%) in circulating triglyceride concentrations.

**Vasodilator Effects**

Prior to estrogen administration, endothelium-dependent vasodilation to ACh was significantly impaired in obese Zuckers compared to the lean Zuckers. $E_{max}$ was reduced and $EC_{50}$ was significantly increased in the OC group compared to the LC group (Figures 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-derived relaxing factors other than NO (e.g. EDHF, prostacyclin), increased endothelium-derived
contractile factors (e.g. endothelin, thromboxane A\(_2\), PGH\(_2\)), increased superoxide production, or a combination of these mechanisms. Since, 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 EDHF. 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 NOSIII 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 Zuckers by significantly reducing EC\(_{50}\) in the OE group. NOSIII protein expression was significantly lower in the OC group compared to LC group, but estrogen administration profoundly increased expression of NOSIII in the aortic rings of OE group. These findings are consistent with the estrogen-mediated leftward shift in EC\(_{50}\) of the ACh curves in the OE group. Estrogen treatment also shifted the ACh curve leftward in the LE group via a reduction in EC\(_{50}\) despite a lack of an effect by estrogen on NOSIII protein expression. These findings suggest that the improved endothelium-dependent vasodilation in response to ACh in the LE and OE groups occurred via 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 than compared to lean Zucker rats resulting in less sexual receptivity in the obese rats (31). Sexual behavior in the obese
Zucker rats following 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 NOSIII (a genomic based mechanism for enhanced endothelial function) or increases NOSIII activity rapidly by a non-genomic mechanism (6). This enhanced NOSIII activity does not involve increases in endothelial cytosolic Ca$^{2+}$ (6). Recently, Haynes et al. demonstrated that membrane estrogen receptor activation increased NOSIII activity through a PI3-kinase–Akt-dependent pathway resulting in phosphorylation of NOSIII and enhanced activity (13). 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 Vedemikov 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 EC$_{50}$, 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) gender differences in responsiveness to estrogen treatment, (2) higher baseline estrogen concentrations in the OC group compared to the LC group, (3) the change in estradiol concentrations following 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.

Summary

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, since the same relationship existed with the pre-treatment of L-NAME prior to generation of the PE curve. Furthermore, this finding was not a result of a cyclooxygenase-dependent factor, because preincubation of the rings with INDO similarly affected vascular responses to PE in both 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 following initiation of HRT in postmenopausal women with underlying heart disease.
ACKNOWLEDGMENTS

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REFERENCES


17. Jiang C, Sarrel PM, Lindsay DC, Poole-Wilson PA, and Collins P. Endothelium-
    independent relaxation of the rabbit coronary artery by 17β-estradiol in vitro. Br J

18. Kanashiro CA, Cockrell KL, Alexander BT, Granger JP, and Khalil RA. Pregnancy-
    associated reduction in vascular protein kinase C activity rebounds during inhibition of

    Postmenopausal estrogens and risk of myocardial infarction in diabetic women. Diabetes

    and Shin EK. Vascular effects of estrogen in Type II diabetic postmenopausal women. J

    Risk. 4: 70-75, 1997.

    TP, Rhodes CJ and King GL. Regulation of endothelial constitutive nitric oxide
    synthase gene expression in endothelial cells and in vivo. A specific vascular action of

23. Kurtz TW, Morris RC, and Pershadsingh HA. The Zucker fatty rat as a genetic model of

24. Laakso M. Hyperglycemia and cardiovascular disease in Type 2 diabetes. Diabetes. 48:


FIGURE LEGENDS

Figure 1. Maximal tension development in response to a 60 mM bolus dose of KCl in LC and LE groups was significantly lower compared to the OC group (* p<0.05). Estrogen treatment decreased the maximal contractile response to KCl for the obese and lean Zuckers; however, this reduction was only significant for the OE group (* p<0.001).

Figure 2. The maximal tension development (E_{max}) to phenylephrine (10^{-9} M to 10^{-5} M) was significantly greater in the OC group compared to LC and LE groups. The dose concentration of PE required to achieve 50% of the maximal tension (EC_{50}) was significantly lower in the OC group compared to LC group. Two to three weeks of estrogen treatment significantly reduced E_{max} in the OE group, and shifted EC_{50} to a lower concentration in both LE and OE groups.

Figure 3. Pre-treatment of the aortic rings with L-NAME, a NOSIII inhibitor, resulted in a significant increase in E_{max} and decrease in EC_{50} in both lean (A) and obese (B) Zuckers.

Figure 4. Preincubation with 10^{-4}M indomethacin, a cyclooxygenase enzyme inhibitor, significantly and similarly reduced E_{max} and increased EC_{50} in lean Zucker rats (*, significantly different than without INDO, p<0.05).

Figure 5. Vasodilation in response to acetylcholine (ACh), an endothelium-dependent vasodilator, was significantly impaired in the OC group compared to LC group. Estrogen treatment significantly improved vasodilation to ACh in the OE group by decreasing the drug concentration required to achieve 50% of maximal relaxation. On the other hand, estrogen
treatment significantly reduced the maximal capacity of the aortic rings of the OE and LE groups to dilate in response to ACh.

**Figure 6.** Vasodilation in response to acetylcholine (ACh), an endothelium-dependent vasodilator, was significantly impaired in the OC group compared to LC group. Estrogen treatment significantly reduced the maximal capacity of the aortic rings of the OE and LE groups to dilate in response to ACh (top panel). Estrogen treatment significantly improved vasodilation to ACh in the OE group by decreasing the drug concentration required to achieve 50% of maximal relaxation (bottom panel). (*, significantly different than LC group; †, significantly different than LE group; § significantly different than OC group)

**Figure 7.** Endothelial-derived nitric oxide synthase (ecNOS or NOSIII) protein expression was quantified by Western Blot analysis. NOSIII protein expression was significantly lower in the OC group compared to LC and LE groups (*, p<0.05). Estrogen treatment significantly increased NOSIII protein expression in the OE group (**, p<0.05) such that protein expression for the OE group was similar to LC and LE groups of Zuckers.

**Figure 8.** Western blot analysis and SDS-PAGE. Representative bands for NOSIII (140 kDa protein) protein expression for LC, LE, OC, and OE groups of Zucker rats.
TABLES

Table 1. Anthropometric Characteristics and Blood Chemistry Profile of Experimental Groups.

<table>
<thead>
<tr>
<th></th>
<th>Lean Control</th>
<th>Lean + E&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Obese Control</th>
<th>Obese+ E&lt;sub&gt;2&lt;/sub&gt;</th>
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<tbody>
<tr>
<td><strong>Age, weeks</strong></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><strong>Pre-Weight, g</strong></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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<tr>
<td><strong>Post-Weight, g</strong></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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<td><strong>Food Ingested, g</strong></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>
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<td><strong>Glucose, mg/dl</strong></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>
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<tr>
<td><strong>Estradiol, pg/ml</strong></td>
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<td>21.8±6.6</td>
<td>30.5±6.3†</td>
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<tr>
<td><strong>Progesterone, pg/ml</strong></td>
<td>4.1±0.6</td>
<td>5.5±0.7</td>
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<td><strong>Triglycerides, mg/dl</strong></td>
<td>68.4±15.0</td>
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<td>204.7±37.6*</td>
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<td><strong>Total Cholesterol, mg/dl</strong></td>
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<td><strong>Insulin, mg/dl</strong></td>
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<td>1.8±0.5</td>
<td>39.6±13.2*</td>
<td>16.4±6.3*</td>
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Table values represent mean±SEM.
* obese vs. lean Zuckers, p<0.05
† estradiol vs. control, p<0.05
Table 2. Phenylephrine dose-response curve parameters for experimental groups

<table>
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<tr>
<th></th>
<th>Lean Control</th>
<th>Lean + E&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Obese Control</th>
<th>Obese + E&lt;sub&gt;2&lt;/sub&gt;</th>
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<td>OC</td>
<td>OE</td>
</tr>
<tr>
<td>No L-NAME</td>
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<td>n=10</td>
<td>n=8</td>
<td>n=10</td>
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<tr>
<td><strong>E&lt;sub&gt;max&lt;/sub&gt;, mg</strong></td>
<td>1827±96</td>
<td>1855±67</td>
<td>2162±101§</td>
<td>1811±53†</td>
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<tr>
<td><strong>-log EC&lt;sub&gt;50&lt;/sub&gt;, M</strong></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>
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<tr>
<td>L-NAME</td>
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<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
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<tr>
<td><strong>E&lt;sub&gt;max&lt;/sub&gt;, mg</strong></td>
<td>2348±185‡</td>
<td>2513±97‡</td>
<td>2698±85‡§</td>
<td>2435±94†‡</td>
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<tr>
<td><strong>-log EC&lt;sub&gt;50&lt;/sub&gt;, M</strong></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>
</tbody>
</table>

Table values represent mean±SEM.
* Significantly different than lean counterparts, p<0.05
† Significant estradiol effect, p<0.05
‡ L-NAME vs. No L-NAME, p<0.05
§ OE group vs. OC, LE, and LC groups, p<0.05
Maximal Tension Development to 60mM KCl

- **Control**
- **Estrogen**

**Tension (mg)**

- Lean
- Obese

The graph shows a comparison of tension development for lean and obese conditions with and without estrogen treatment. The estrogen treatment results in a statistically significant increase in tension development, as indicated by the * symbol.
Influence of Indomethacin (INDO) on Maximal Contractile Response ($E_{\text{max}}$) to Phenylephrine: Control vs. Estrogen

Influence of Indomethacin (INDO) on Vascular Sensitivity to Phenylephrine: Control vs. Estrogen
$E_{\text{max}}$: Response to ACh

Lean Obese

% Relaxation

Control

Estrogen

*†§

Treatment

Lean Obese

$EC_{50}$: Response to ACh

Lean Obese

-log $[ACh, M]$
NOSIII Protein Expression

% of Obese Control Group

Lean

Obese

Control

Estrogen

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R2
NOSIII
140 kDa