Loss of ovarian function in the VCD mouse-model of menopause leads to insulin resistance and a rapid progression into the metabolic syndrome

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Romero-Aleshire MJ, Diamond-Stanic MK, Hasty AH, Hoyer PB, Brooks HL. Loss of ovarian function in the VCD mouse-model of menopause leads to insulin resistance and a rapid progression into the metabolic syndrome. Am J Physiol Regul Integr Comp Physiol 297: R587–R592, 2009. First published May 13, 2009; doi:10.1152/ajpregu.90762.2008.—Factors comprising the metabolic syndrome occur with increased incidence in postmenopausal women. To investigate the effects of ovarian failure on the progression of the metabolic syndrome, female B6.C3F1 mice were treated with 4-vinylcyclohexene diepoxide (VCD) and fed a high-fat (HF) diet for 16 wk. VCD destroys preantral follicles, causing early ovarian failure and is a well-characterized model for the gradual onset of menopause. After 12 wk on a HF diet, VCD-treated mice had developed an impaired glucose tolerance, whereas cycling controls were unaffected [12 wk AUC HF mice 13,455 ± 643 vs. HF/VCD 17,378 ± 1140 mg/dl/min, P < 0.05]. After 16 wk on a HF diet, VCD-treated mice had significantly higher fasting insulin levels (HF 5.4 ± 1.3 vs. HF/VCD 10.1 ± 1.4 ng/ml, P < 0.05) and were significantly more insulin resistant (HOMA-IR) than cycling controls on a HF diet (HF 36.2 ± 16.7 vs. HF/VCD 113.1 ± 19.6 mg/dl/μU/ml, P < 0.05). All mice on a HF diet gained more weight than mice on a standard diet, and weight gain in HF/VCD mice was significantly increased compared with HF cycling controls. Interestingly, even without a HF diet, progression into VCD-induced menopause caused a significant increase in cholesterol and free fatty acids. Furthermore, in mice fed a standard diet (6% fat), insulin resistance developed 4 mo after VCD-induced ovarian failure. Insulin resistance following ovarian failure (menopause) was prevented by estrogen replacement. Studies here demonstrate that ovarian failure (menopause) accelerates progression into the metabolic syndrome and that estrogen replacement prevents the onset of insulin resistance in VCD-treated mice. Thus, the VCD model of menopause provides a physiologically relevant means of studying how sex hormones influence the progression of the metabolic syndrome.

estrogen; cholesterol; glucose; insulin

BY THE YEAR 2025, POSTMENOPAUSAL aged women will make up 20% of the U.S. population. In addition the incidence of obesity is also increasing. Obesity particularly contributes to the metabolic syndrome, which is an increasingly prevalent disorder that affects ~47 million people in the United States (2) and involves a clustering of risk factors for cardiovascular disease and type 2 diabetes. The incidence of diabetes seems to be slightly lower in women than men throughout most of life (37); however, as estrogen levels drop, the estrogen/androgen ratio changes in postmenopausal women, and a change in this ratio may affect cardiovascular disease and the metabolic syndrome (12, 14, 15). Indeed, postmenopausal women display an increased insulin resistance compared with premenopausal women, predisposing them to the development of diabetes.

In animals, ovariectomy (OVX), or the surgical excision of the ovaries, has been the most commonly used model for menopause research. With this approach, ovarian estrogen production ceases suddenly, and there is no androgen production from residual ovarian tissue. Recently, however, an ovary-intact mouse model of menopause has been developed using the occupational chemical 4-vinylcyclohexene diepoxide (VCD) (18, 19, 23). Repeated daily dosing with VCD selectively destroys primordial and primary follicles in ovaries of mice and rats by accelerating the natural process of follicular atresia (11, 30, 33). Evidence for VCD’s lack of toxicity to other tissues or organ systems was published in early work by the National Toxicology Program. In these studies, mice and rats were repeatedly exposed to VCD for 2 years, and other than skin lesions at the site of repeated dermal application, the only notable effects were in female ovaries, and no other systems were impacted by this chronic exposure (1).

To test the potential negative impact of gradual estrogen loss on the progression of the metabolic syndrome in the female VCD-treated mouse, we fed both VCD-treated and placebo control mice a high-fat or a standard diet over a period of 16 wk. The development and progression of the metabolic syndrome were assessed by measuring parameters associated with the metabolic syndrome; specifically, glucose tolerance, insulin levels, weight gain, and lipid profiles. Studies here will demonstrate that ovarian failure (menopause) accelerates progression into the metabolic syndrome and that estrogen replacement prevents the onset of insulin resistance in VCD-treated mice, strengthening the role of the VCD model of menopause as a physiologically relevant means of studying how sex hormones impact disease progression.

MATERIALS AND METHODS

Animals. Twenty-one-day-old female B6.C3F1 mice were purchased from Harlan Laboratories. Animals were housed in standard University Animal Care cages under standard light cycles and humidity. Animals were allowed ad libitum access to food and water, and body weight was measured biweekly. All experiments were approved by the University of Arizona Institutional Animal Care and Use Committee.

Induction of metabolic syndrome. After a 3-day acclimation period in the animal facility, 24-day-old mice were switched from the standard University Animal Care diet (7013; Harlan Teklad) to a high-fat diet (D12331; Research Diets). Control and VCD-treated mice were fed the standard diet, which consisted of 18% protein and 6% fat. HF and HF/VCD mice were fed the high-fat diet, which...
comprised 16.4% protein and 58% fat. Animals were fed their respective diets for the entire duration of the study. At the end of the study, animals were euthanized and fat pads were excised and weighed to compare abdominal fat pad mass.

VCD treatment. After 1 wk of acclimation in the animal facility, 28-day-old mice were weighed and injected daily intraperitoneally (i.p.) with VCD (V3630; Sigma) at a concentration of 160 mg/kg for 17 consecutive days (19). Control animals were injected with a sesame oil vehicle control. After mice were dosed, vaginal cytology was monitored daily to determine cessation of cyclicity. Mice were considered acyclic after 15 consecutive days without estrous (23). After mice were killed the ovaries were excised and trimmed of remaining fat tissue. Ovaries were fixed in Bouin’s fixative, paraffin embedded, and 4-μm serial sections were prepared. Every 20th section was retained for ovarian follicle counts to avoid double counting of antral follicles. Sections were mounted and stained with hematoxylin and eosin, and primordial, primary, secondary, and antral follicles were counted as previously described (23). In a separate study, hormone replacement was achieved in a cohort of VCD-treated animals by subcutaneous implants, which consisted of 2.5 cm of Silastic medical tubing (508–011; Dow Corning) and 180 μg/ml 17β-estradiol (E-8875; Sigma) in sesame oil. Approximately every 4 wk, the old implant was removed and a fresh one was inserted to maintain adequate circulating levels (circulating levels of 17β-estradiol measured 1 wk after implant surgery were ~53.99 + 4.3 pg/ml). In hormone replacement-treated animals, glucose tolerance tests were performed 184 days post-VCD treatment, as described below.

Intraperitoneal glucose tolerance test. Intraperitoneal glucose tolerance tests (IPGTT) were performed 8, 12, and 16 wk after the onset of VCD dosing. Mice were fasted for 4 h before glucose challenge. Animals were injected intraperitoneally with glucose at a concentration of 1 g/kg body wt. Approximately 15 μl of whole blood was drawn from the tip of the tail vein, and glucose was measured with the CardioCheck PA Blood Testing Device (2568; HealthCheck Systems). Blood was collected at 0, 15, 30, 60, and 120 min after glucose injection. Glucose tolerance comparisons were made by measuring the area under the glucose clearance curve (AUCg) using SigmaPlot 2001 software.

Blood glucose and plasma assays. Mice were fasted for 4 h, euthanized by CO2 inhalation, and blood was drawn by cardiac puncture and collected in heparinized syringes. Blood glucose levels were measured with the same glucose meter as in the IPGTT experiments. Whole blood was then centrifuged, and the plasma was extracted and stored at −80°C for future study. Plasma insulin was measured with a rat/mouse insulin ELISA kit (EZRMI-13K; Linco Research) using the provided protocol. The homeostasis model assessment of insulin resistance (HOMA-IR) was used to measure fasting whole body insulin resistance using the calculation [(fasting blood glucose (mg/dl) × fasting plasma insulin (μU/ml))/405] (22).

Plasma triglycerides and cholesterol were measured using kits from Raichem following the manufacturer’s instructions (84098 and 80015; Raichem). Nonesterified fatty acids were measured with the NEFA-C kit (994-75409; Wako Chemicals).

Statistics. Data were analyzed using Student’s t-test or one-way ANOVA followed by a Tukey’s post hoc test to identify differences between groups. Significance was determined as P < 0.05. Results are represented as means ± SE.

RESULTS

VCD-treated mice had impaired glucose tolerance after 12 weeks on a high-fat diet. To investigate the effects of ovarian failure on the progression of the metabolic syndrome, female B6C3F1 hybrid mice were treated with VCD and fed a high-fat (HF) diet for up to 16 wk. The VCD-treated group (HF/VCD) was injected with VCD for 15 consecutive days to cause ovarian failure, and the cycling control group, (HF), was injected with vehicle. Additional groups of mice were fed a standard diet (6% fat) for comparison and were separated into VCD-treated and vehicle-treated cycling control group. Glucose tolerance tests were performed after 12 wk on the respective diets. Significant differences were observed in the high-fat fed mice at this 12-wk time point (Fig. 1); VCD-treated mice had developed an impaired glucose tolerance; in contrast, cycling control mice were unaffected and had a normal glucose tolerance (HF/VCD AUC 17,378 ± 1,140 vs. HF AUC 13,455 ± 643 mg/dl/min, P < 0.05). The impaired glucose tolerance in the HF/VCD mice was not observed at the earlier 8-wk time point (data not shown). Glucose tolerance tests were performed again after 16 wk on the respective diets, and at this time point both VCD-treated and cycling control mice on the high-fat diet had developed an impaired glucose tolerance (Fig. 2). In contrast, all animals on a standard diet (6% fat) demonstrated normal glucose clearance at all time points (8, 12, and 16 wk).

VCD-treated mice on a high-fat diet gained more weight than cycling controls on a high-fat diet. Weight gain was monitored in all groups twice weekly. All mice on a high-fat diet weighed more than mice on a standard diet (Table 1). However, VCD-treated mice on a high-fat diet increased their weight more rapidly than all other groups, such that HF/VCD mice were significantly heavier than HF cycling control mice at the 12 wk and the 16 wk time point (P < 0.05).

High-fat diet induced an increase in abdominal fat weight. One of the characteristics of the metabolic syndrome is increased intra-abdominal obesity. Reportedly, estrogen deple-
tion also promotes abdominal fat accumulation. After 16 wk on a high-fat diet, all mice were killed, and abdominal fat pads were weighed (Fig. 3). Both groups of mice on a high-fat diet (VCD-treated and cycling mice) had a significant increase in fat pad weight compared with mice on a standard diet (mean increase in fat pad weight in HF/VCD mice 3.6 ± 0.2 g vs. mean increase in fat pad weight in HF mice 4.2 ± 0.1 g; P < 0.05 vs. mice on a standard diet). However, surprisingly, given that HF/VCD mice were significantly heavier than HF cycling mice, there was a trend for the fat pads in the HF cycling mice to be heavier compared with fat pads from HF/VCD mice (P = 0.052).

VCD-treated mice became hyperinsulinemic and insulin resistant compared with controls. After 16 wk on a high-fat diet VCD-treated (HF/VCD) and vehicle-treated mice (HF) had high circulating levels of cholesterol and free fatty acids (FFA), and also had high fasting blood glucose levels compared with mice on a standard diet. However, there was no significant difference between the HF/VCD and the HF groups for these three parameters (Table 2). In contrast, VCD-treated mice on the high-fat diet had significantly higher circulating insulin levels than cycling mice on a high-fat diet (Table 2). HOMA-IR is a measure of whole body insulin resistance. As shown in Fig. 4, after 16 wk on a high-fat diet, VCD-treated mice were significantly more insulin resistant than cycling control mice on a high-fat diet (HF/VCD 113.1 ± 22.7 vs. HF 56.9 ± 16.7 mg/dl·μU/ml, P < 0.05).

Interestingly, VCD-treated mice on a standard diet had significantly higher circulating FFA and cholesterol levels compared with control mice on a standard diet (Table 2), suggesting that ovarian failure was causing significant alterations in blood lipids.

Ovarian failure contributes to the onset of the metabolic syndrome. To determine whether ovarian failure alone led to an onset of insulin resistance, an additional study was performed in VCD-treated mice on a standard diet. Three groups of mice were used, all on a standard diet; two VCD-treated groups and a control vehicle-treated group. Mice in one VCD-treated group were also given 17β-estradiol, via a subcutaneous implant (VCD/E2). As shown in Fig. 5, mice that had undergone ovarian failure (VCD) had an impaired glucose tolerance, whereas cycling mice had normal responses. VCD-treated mice that underwent ovarian failure but received continuous 17β-estradiol infusion had a normal glucose tolerance. These data suggest that estrogen replacement prevented the development of the metabolic syndrome.

Table 2. Combined effect of ovarian status and diet on blood glucose, lipid profiles and insulin

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<th>Standard Diet</th>
<th>High-Fat Diet</th>
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<tr>
<td></td>
<td>Cycling (Con)</td>
<td>VCD</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>117±10</td>
<td>135±4</td>
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<tr>
<td>Cholesterol, mg/dl</td>
<td>148±4</td>
<td>163±6*</td>
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<tr>
<td>FFA, mEq/L</td>
<td>1.9±0.04</td>
<td>2.9±0.5*</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>113±8</td>
<td>108±11</td>
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<tr>
<td>Insulin, ng/ml</td>
<td>0.8±0.1</td>
<td>0.8±0.1</td>
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Mice were fed a high-fat or a standard diet for 16 wk and sacrificed, and blood/plasma was collected. All mice were fasted for 4 h prior to measurement. FFA, free fatty acid. *P < 0.05 vs. cycling mice; #P < 0.05 vs. cycling mice on high-fat diet.

Table 1. Body weights after 8, 12, and 16 wk of a high-fat or a standard diet

<table>
<thead>
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<th>Standard Diet</th>
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<tr>
<td></td>
<td>Cycling (Con)</td>
<td>VCD</td>
</tr>
<tr>
<td>8-wk weight, g</td>
<td>25.3±0.8</td>
<td>24.8±0.7</td>
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<tr>
<td>12-wk weight, g</td>
<td>30.1±1.3</td>
<td>30.7±1.4</td>
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<tr>
<td>16-wk weight, g</td>
<td>34.3±1.5</td>
<td>36.3±1.8</td>
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Mice were fed a high-fat (HF) diet for 16 wk. Mice were weighed biweekly throughout the duration of the study. VCD, 4-vinylcyclohexene diepoxide. #P < 0.05 vs. cycling mice on a high-fat diet.
mented an impaired glucose tolerance following ovarian failure in VCD-treated mice.

DISCUSSION

Menopause is defined as the cessation of menstrual cyclicity. It is preceded by a period called perimenopause, when circulating 17β-estradiol levels decline gradually and cycle lengths are variable (29). Perimenopause can last up to 10 years in women. After menopause, the ovaries cease to produce antral follicular estrogen, and 17β-estradiol levels decrease to undetectable levels. Furthermore, residual ovarian tissue continues to produce androgens (34) and is thought to be responsible for 25–35% of postmenopausal circulating androgens (16). However, the biological impact of postmenopausal ovarian androgen production on health is widely debated (4).

The purpose of this study was to determine whether progression through menopause (ovarian failure in mice) increased the rate of onset of insulin resistance and the metabolic syndrome. We used a new model of ovarian failure in mice, the VCD mouse model of menopause, which has been well characterized in B6C3F1 hybrid mice. VCD treatment in mice increased the rate of onset of insulin resistance and the metabolic progression through menopause (ovarian failure in mice) in rodents. Estrogen exerts an important androgenic (27). Thus, the VCD model more closely approximates the natural progression of menopause in women in which the gradual depletion of estrogen precedes menopause and the ovarian tissue is retained.

In this study, we combined the VCD model of menopause with a high-fat diet. Data presented here demonstrate that VCD-treated mice fed a high-fat diet developed impaired glucose tolerance after only 12 wk, 4 wk earlier than cycling controls on the same diet. These data suggest that the loss of ovarian function increased the onset of high-fat diet-induced insulin resistance.

Although it is well known that a high-fat diet when fed to rodents initiates progression into the metabolic syndrome, typically diet-induced metabolic syndrome research has been conducted in male C57BL/6 mice since these mice become rapidly obese, hyperinsulinemic, and hyperglycemic (24, 31). In contrast diet-induced metabolic syndrome is rarely studied in female mice despite reports of sex differences in weight gain, blood glucose, and insulin levels (32).

A significantly greater weight gain was observed in the VCD-treated mice fed a high-fat diet compared with cycling control animals on a high-fat diet. These results are similar to what is seen in humans; following menopause, women tend to gain weight and body fat (9). However, there is conflicting data from ovarioctomized animals; some studies observe an increase in body weight following ovariectomy (6, 36) compared with intact animals, whereas other studies do not (7, 13).

Our studies demonstrated that after 16 wk of a high-fat diet, VCD-treated mice had significantly higher circulating insulin levels than cycling controls and were significantly more insulin resistant. Thus, estrogen depletion in the VCD model expedited the development of the metabolic syndrome and exacerbated insulin abnormalities. This is in agreement with studies in both rodents and humans that have shown that sex hormones have also been implicated in insulin action. In humans, increased insulin sensitivity has been reported in females via increased insulin-mediated glucose uptake in muscles and insulin-mediated FFA reuptake (17, 25). Studies in Wistar rats demonstrated that females needed less insulin in response to an oral administration of 17β-estradiol on glucose tolerance following ovarian failure.
glucose challenge compared with males (8). In postmenopausal women, hormone replacement therapy attenuated insulin resistance (10). Furthermore, in gonadectomized male rats, estrogen treatment elevated insulin sensitivity (35). Taken together these data infer a beneficial relationship between estrogen and insulin sensitivity.

We confirmed this beneficial relationship by replacing estrogen in an additional cohort of VCD-treated animals. When estrogen was replete the impaired glucose tolerance observed in the VCD-treated mice was attenuated. Interestingly, the incidence of new cases of type 2 diabetes is lower in postmenopausal women receiving hormone replacement therapy than in placebo-treated women (12). In addition, hormone replacement therapy improves insulin resistance in postmenopausal women with impaired fasting glucose (21, 28). Overall, these observations suggest that progression into the metabolic syndrome is influenced by menopausal status.

One interesting observation from this study was that VCD-treated mice on a standard chow diet (6% fat) had elevated circulating FFA levels and elevated cholesterol compared with their control group (cycling controls). Indeed, in the human population, there appears to be a relationship between estrogen depletion and increased FFA levels, as FFA levels increase significantly in ovariectomized women (26). However, little is known about how estrogen depletion combined with obesity influences FFA levels.

Reproductive health, ovarian cyclicity, and insulin resistance are known to be linked in women. Insulin resistance with a compensatory hyperinsulinemia commonly occurs in women with polycystic ovary syndrome (PCOS). Women with PCOS also tend toward obesity and have menstrual dysfunction. Women with PCOS have an increased risk of developing type 2 diabetes mellitus and cardiovascular disease (5, 20). The VCD model of menopause does retain androgenic ovarian tissue after ovarian failure is complete (27); however, the contribution of androgens in this model needs further study.

Perspectives and Significance

In the United States, ~4,000 women enter menopause each day (3). Demographic studies on the average age of menopause in the United States have shown that it has increased from ~45 years in 1850, to 51 in 1995. However, the life expectancy of women has increased from ~45 in 1850 to around 80 in 2006 (3). Thus, the life span in women has shifted such that more than 30% of a woman’s lifetime will be postmenopausal. The consequence of this shift is that many age-related diseases are increasing in incidence and need to be investigated in relevant animal models to understand the effect of menopause on disease risk, presentation, and progression. We demonstrate here that menopause increases the rate of progression into the metabolic syndrome and that estrogen replacement attenuates this progression. The data highlight that the VCD mouse model of menopause is ideally suited for designing future studies related to perimenopausal and postmenopausal physiology. Future studies using this powerful approach will provide important insight into the mechanisms behind the serious health risks associated with the metabolic syndrome in postmenopausal women, such as increased risk for cardiovascular disease and diabetic complications.

GRANTS

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15. Labrie F, Luu-The V, Labrie C, Belanger A, Simard J, Lin SX, Pelletier G. Inhibition of breast cancer and other roles of androgens and their precursor androgenic ovarian tissue after ovarian failure is complete (27); however, the contribution of androgens in this model needs further study.