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 B6C3F1 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 afflicts ~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 increase in women than men throughout most of life (37); however, as estrogen levels drop, the estrogen/androgen 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 B6C3F1 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 CardiCheck 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 groups. 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-
MURINE MODEL OF POSTMENOPAUSAL METABOLIC SYNDROME

Fig. 2. Impaired glucose tolerance occurs in cycling female mice after 16 wk on a high-fat diet. Intraperitoneal glucose tolerance tests (IPGTT) were performed in all groups after 16 wk on a high-fat (58%) or a standard diet (6%). Glucose tolerance curves were plotted, and AUCg were calculated, n = 3 in each group. For IPGTT curves, triangles denote control cycling mice; rectangles denote VCD-treated mice; solid symbols denote standard diet; open symbols denote high-fat diet. Results are expressed as means ± SE. *P < 0.05 vs. control cycling mice on a standard diet.

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

<table>
<thead>
<tr>
<th>Metric</th>
<th>Standard Diet</th>
<th>High-Fat Diet</th>
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<tbody>
<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>
</tr>
<tr>
<td>12-wk weight, g</td>
<td>30.1±1.3</td>
<td>30.7±1.4</td>
</tr>
<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.

Fig. 3. Effect of ovarian status and diet on abdominal fat pad weight. After 16 wk on a high-fat (58%) or standard diet (6%), all mice were euthanized, and abdominal fat pads were excised and weighed. Results are expressed as means ± SE; n = 6 in each group.

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

<table>
<thead>
<tr>
<th>Metric</th>
<th>Standard Diet</th>
<th>High-Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycling (Con)</td>
<td>VCD</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>117±10</td>
<td>135±4</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>148±4</td>
<td>163±6*</td>
</tr>
<tr>
<td>FFA, mEq/L</td>
<td>1.9±0.04</td>
<td>2.9±0.5*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>113±8</td>
<td>108±11</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.8±0.1</td>
<td>0.8±0.1</td>
</tr>
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</table>

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.

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 develop-
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gen production on health is widely debated (4).

DISCUSSION

Menopause is defined as the cessation of menstrual cyclicity. It is preceded by a period called perimenopause, when circu-
ulating 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 unde-
tectable levels. Furthermore, residual ovarian tissue continues
to produce androgens (34) and is thought to be responsible for
25–35% of postmenopausal circulating androgens (16). How-
ever, the biological impact of postmenopausal ovarian andro-
gen production on health is widely debated (4).

The purpose of this study was to determine whether pro-
gression through menopause (ovarian failure in mice) in-
creased 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 char-
acterized in B6C3F1 hybrid mice. VCD treatment in mice
causes ovarian failure in a gradual manner and thus is accom-
pained by a gradual depletion in circulating estrogen levels and
an increase in circulating FSH levels (19). As VCD does not
target larger follicles, only primary and primordial follicles,
VCD-treated mice continue to ovulate normally until no more
follicles can be recruited. Thus, ovarian follicular depletion in
the VCD-treated mouse is gradual. As with women undergoing
perimenopause, VCD-treated mice show increased levels of
FSH (23), (27), as well as declining levels of estrogen and
irregular estrous cycles (18, 38), as they become follicle-
depleted. Additionally, unlike OVX animals generally used to
model menopause, the VCD-treated animal retains residual
ovarian tissue (23), and this tissue was recently shown to be
androgenic (27). Thus, the VCD model more closely approx-
imates 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
insulin resistance 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 ovarietomized animals; some studies observe an in-
crease 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 reuptake in muscles and insulin-
mediated FFA reuptake (17, 25). Studies in Wistar rats dem-
onstrated that females needed less insulin in response to an oral

**Fig. 4.** Effect of ovarian status on diet-induced insulin resistance. Fasting
insulin and glucose values were determined at 16 wk to calculate HOMA-IR
values. Results are expressed as means ± SE.

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**Fig. 5.** Effect of 17β estradiol on glucose tolerance following ovarian failure.
Mice were dosed with vehicle control or 4-vinylcyclohexene diepoxide (VCD)
(day 1). Silastic implants containing 17β estradiol (180 mg/ml) was inserted
subcutaneously into one group of VCD-treated mice. After 26 wk, intraperi-
toneal glucose tolerance tests (IPGTT) were performed. Glucose tolerance
curves were plotted and AUCg were calculated. For IPGTT curves, rectangles
denote VCD-treated mice; triangles denote VCD-treated mice + 17β estradiol;
diamonds denote control cycling mice. Results are expressed as means ± SE.

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denote VCD-treated mice; triangles denote VCD-treated mice + 17β estradiol;
diamonds denote control cycling mice. Results are expressed as means ± SE.
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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REFERENCES


