Hormonal status affects the progression of STZ-induced diabetes and diabetic renal damage in the VCD mouse model of menopause

Maggie Keck, Melissa J. Romero-Aleshire, Qi Cai, Patricia B. Hoyer, and Heddwen L. Brooks

Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona

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Keck M, Romero-Aleshire MJ, Cai Q, Hoyer PB, Brooks HL. Hormonal status affects the progression of STZ-induced diabetes and diabetic renal damage in the VCD mouse model of menopause. Am J Physiol Renal Physiol 293: F193–F199, 2007. First published March 27, 2007; doi:10.1152/ajprenal.00022.2007.—Changes in the estrogen/testosterone balance at menopause may negatively influence the development of diabetic kidney disease. Furthermore, recent studies suggest that changes in hormone levels during perimenopause may influence disease development. Injection of 4-vinylcyclohexene diepoxide (VCD) in B6C3F1 mice induces gradual ovarian failure, preserving both the perimenopausal (peri-ovarian failure) and menopausal (post-ovarian failure) periods. To address the impact of the transition into menopause on the development of diabetes and diabetic kidney damage, we used streptozotocin (STZ)-induced diabetes in the VCD model of menopause. After 6 wk of STZ-induced diabetes, blood glucose was significantly increased in post-ovarian failure (post-OF) diabetic mice compared with cycling diabetic mice. In peri-ovarian failure (peri-OF) diabetic mice, blood glucose levels trended higher but were not significantly different from cycling diabetic mice, suggesting a continuum of worsening blood glucose across the menopausal transition. Cell proliferation, an early marker of damage in the kidney, was increased in post-OF diabetic mice compared with cycling diabetic mice, as measured by PCNA immunohistochemistry. In post-OF diabetic mice, mRNA abundance of early growth response-1 (Egr-1), collagen-4α1, and matrix metalloproteinase-9 were increased and 3β-hydroxysteroid dehydrogenase 4 (3β-HSD4) and transforming growth factor-β2 (TGF-β2) were decreased compared with cycling diabetic mice. In peri-OF diabetic mice, mRNA abundance of Egr-1 and 3β-HSD4 were increased, and TGF-β2 was decreased compared with cycling diabetic mice. This study highlights the importance and utility of the VCD model of menopause, as it provides a physiologically relevant system for determining the impact of the menopausal transition on diabetes and diabetic kidney damage.

diabetes; 3β-HSD4; perimenopause; real-time PCR; estrogen

Address for reprint requests and other correspondence: H. Brooks, Dept. of Physiology, College of Medicine, 1501 N Campbell Ave, Univ. of Arizona, Tucson, AZ 85724-5051 (e-mail: brooksh@email.arizona.edu).

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further suggest that the perimenopausal period may be important in the development and progression of diabetic renal disease. This study highlights the utility of the VCD mouse model of menopause in the study of diabetic renal disease across the menopausal transition.

METHODS

Animals. Twenty-eight-day-old female B6C3F1 mice were used in this study. Animals were housed in standard cages in the animal facility of the Arizona Health Sciences Center under National Institutes of Health guidelines and had ad libitum access to regular food and water. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Induction of ovarian failure. VCD (Sigma, V3630) was administered via intraperitoneal injection at a dose of 160 mg/kg body wt using a dosing standard of 2.5 ml/kg body wt for 15 consecutive days to induce gradual ovarian failure (13, 20). Mice were killed 6 wk after STZ injection. Mice were fasted for 4 h before the end of VCD dosing (Fig. 1: Perimenopausal Study). In both studies, cycling mice (not VCD-injected) were injected with STZ on the same day. Citrate buffer was used as vehicle control. Urine glucose was measured using the CardioCheck PA Blood Testing Device (HealthCheck Systems, SC-25280), followed by biotinylated secondary antibody (1:200 dilution; Zymed, 81-6740) for 30 min at 37°C and horseradish peroxidase-conjugated streptavidin (1:200 dilution; Zymed, 43-4323) for 30 min at 37°C. Labeling was visualized with chromogen diaminobenzidine (Zymed, 00-8011). Coverslips were mounted with Permount mounting solution (Fisher, SP15-100). Positively stained nuclei were counted as a proportion of total nuclei within each of five nonoverlapping fields of view for each section. Each field of view contained ~200 cells.

Real-time PCR. Real-time PCR was performed as previously described (21). Briefly, 2.5 μg of RNA were reverse transcribed with the MLV-Reverse Transcriptase enzyme (Invitrogen, 28025013), and the resulting cDNA was diluted 1:25 to an approximate final concentration of 8 ng/μl. Each real-time PCR reaction contained 5 μl SYBR Green master mix (Stratagene, 600581), 1 μl water, 2 μl diluted cDNA, and 5 pmol each of forward and reverse primer in a total volume of 10 μl. Each reaction was performed in triplicate at 95°C for 5 min and then 95°C for 15 s and 60°C for 30 s for 40 cycles. The RotorGene RG3000 (Corbett Research, San Francisco, CA) sequence detection system was used. Primers were designed to the 3' end of genes of interest using the Primer3 software (30) and are listed in supplemental data A (the online version of this article contains supplemental data). Ct values were used to calculate the expression levels of genes of interest relative to the expression of β-actin mRNA, measured in parallel samples. Analysis was performed as described (11), and results are presented as mean fold change on a base 2 logarithmic scale.

RESULTS

Initiation of menopause. To study the effect of menopause on the development of diabetes and diabetic kidney damage, we combined the STZ model of type 1 diabetes with the VCD model of menopause in B6C3F1 female mice (20). Repeated injections of VCD induce gradual ovarian failure in mice,
which is analogous to menopause in humans. In this study, diabetes was induced using STZ 2 wk after a mouse entered ovarian failure, as shown in Fig. 1. An age-matched cycling mouse (not VCD-injected) was injected with STZ at the same time (Table 1). Mice were killed 6 wk after STZ dosing. Age-matched nondiabetic post-ovarian failure (post-OF) and nondiabetic cycling mice were killed as controls (see Table 1).

Blood glucose in post-ovarian failure diabetic mice. There was a significant increase in blood glucose in all STZ-injected mice compared with control mice (Fig. 2). Furthermore, glucose levels in post-OF diabetic mice were significantly higher than in cycling diabetic mice (336 ± 86 vs. 218 ± 76 mg/dl, \( P < 0.05 \)). There was no significant difference in glucose levels between nondiabetic post-OF mice and cycling control mice (91 ± 23 vs. 79 ± 20, \( P > 0.05 \)).

PCNA immunohistochemistry. Cell proliferation is used as an early indicator of cell stress/damage in the kidney and occurs in the renal glomeruli and tubules in diabetic mice (14, 24, 38). PCNA is a DNA polymerase-associated protein expressed in proliferating cells (39). Immunohistochemistry was performed on kidney tissue sections using an antibody against PCNA. Representative sections of PCNA immunohistochemistry are shown in Fig. 3A. There were significantly more PCNA-positive cells in the renal cortex of diabetic mice compared with nondiabetic mice (Fig. 3B). In addition, there was a further significant increase in PCNA-positive cells in the cortex of post-OF diabetic mice compared with cycling diabetic mice. PCNA staining was primarily in tubular not glomerular cells and was not seen in the medulla of any treatment group.

Real-time PCR in post-ovarian failure diabetic mice. In the kidney the expression of many genes associated with damage, such as collagen 4 and transforming growth factor-\( \beta \), is altered in response to diabetes (32). Thus real-time PCR was performed to determine whether the expression of genes associated with diabetic kidney damage was different depending on hormonal status. Several genes which were differentially expressed in the renal cortex of post-OF diabetic mice compared with cycling diabetic mice were identified. Three main patterns of changes in gene expression emerged, as shown in Fig. 4. Pattern 1: A significant change (increase or decrease) in mRNA abundance in post-OF diabetic mice compared with control mice and cycling diabetic mice, with no change be-

![Image](http://ajprenal.physiology.org/)

**Fig. 2.** Blood glucose measurements in post-ovarian failure diabetic mice. Cycling or post-ovarian failure mice were injected with STZ to induce diabetes. Fasted blood glucose levels were measured 6 wk after STZ treatment. Results are expressed as mean ± SD. Significant difference determined by Student-Newman-Keuls post hoc test following 1-way ANOVA. OF, ovarian failure. See Table 1 for \( n \) in each group.

**Fig. 3.** Effect of diabetes and hormonal status on renal cortex cell proliferation. A: Representative sections from kidney cortex demonstrating PCNA staining (in red); nuclear counterstain with hemotoxylin (blue). a: Control. b: Post-OF. c: Diabetic. d: Post-OF/Diabetic. Magnification \( \times 400 \). B: graph presenting the mean PCNA-positive nuclei as a percentage of total nuclei. PCNA-positive nuclei and total nuclei were counted in 5 nonoverlapping fields of view for each section, with 3 animals in each treatment group. Results are presented as mean ± SD. Significant difference determined by Student Newman-Keuls post hoc test following 1-way ANOVA.

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Traditional views on the regulation of gene expression are shown in Table 1. Descriptions of treatments given to mice. + Indicates a chemical which was injected. Otherwise, mice were injected with appropriate control buffer. Number is the number of mice in each treatment group. VCD, 4-vinylcyclohexene diepoxide; STZ, streptozotocin; OF, ovarian failure.

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Table 1. Description of mouse groups

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Pattern 1: A significant change (increase or decrease) in mRNA abundance in post-OF diabetic mice compared with control mice and cycling diabetic mice, with no change be-
abundance in cycling diabetic mice compared with control mice and a further significant decrease in post-OF diabetic mice (Fig. 4B). mRNA abundance of 3β-hydroxysteroid dehydrogenase 4 (3β-HSD4) fit this pattern, with a 1.39-fold decrease (P < 0.05) in cycling diabetic mice compared with control mice, and a further 1.30-fold decrease (P < 0.05) in post-OF diabetic mice compared with cycling diabetic mice. The mRNA abundance of 3β-HSD4 in post-OF diabetic mice was decreased 1.79-fold (P < 0.05) compared with control mice. Pattern 3: A significant change in mRNA abundance in cycling diabetic mice compared with control mice, and a significant change in the opposite direction between post-OF and cycling diabetic mice such that there was no significant difference between post-OF diabetic and control mice (Fig. 4C). Transforming growth factor-β2 (TGF-β2) fit this pattern; the mRNA abundance of TGF-β2 increased 1.60-fold (P < 0.05) in cycling diabetic mice compared with control mice and decreased 1.59-fold (P < 0.05) in post-OF diabetic mice relative to cycling diabetic mice. There was no significant difference in mRNA abundance of TGF-β2 between post-OF diabetic and control mice. In addition, real-time PCR analysis was performed for the following genes: collagen 1α2, collagen 4α2, estrogen receptor α, fibronectin 1, PCNA, TGF-β1, and TGF-β3. No significant differences in renal cortex mRNA abundance between post-OF diabetic mice and cycling diabetic mice were found. For all of the above genes, there were no significant differences in the mRNA abundance between control and nondiabetic post-OF mice.

Menopause induced weight gain in nondiabetic mice. All mice gained weight throughout the study. Compared with control mice, post-OF nondiabetic mice rapidly gained weight following ovarian failure. Eight weeks post-ovarian failure, post-OF mice weighed significantly more than control mice (Fig. 5). There were no significant differences in weight between any of the other groups of mice.

Initiation of perimenopause. One of the strengths of the VCD model of menopause is that full ovarian failure is preceded by a period of irregular cycling, analogous to perimenopause in human women (13, 20). Thus, in a complementary study diabetes was induced during peri-ovarian failure (see
Fig. 1: Perimenopausal Study); STZ dosing began 3 days after the end of VCD dosing. In contrast to the menopausal study, there was no difference in weight between any of the groups of mice at the end of the study.

**Blood glucose in peri-ovarian failure diabetic mice.** There was a significant increase in blood glucose in all STZ-injected mice compared with control mice (Fig. 6). Glucose levels in peri-OF diabetic mice trended higher but were not significantly different from cycling diabetic mice (238 ± 102 vs. 201 ± 96 mg/dl, P = 0.21). Compared with the menopausal study, glucose levels in peri-OF diabetic mice were significantly different from glucose levels in post-OF diabetic mice (238 ± 102 vs. 336 ± 86 mg/dl, P < 0.05).

**Real-time PCR in peri-ovarian failure diabetic mice.** Real-time PCR was performed comparing renal cortex mRNA abundance from peri-OF diabetic mice to cycling diabetic mice. Primers for genes whose expression was significantly different between post-OF diabetic and cycling diabetic mice (i.e., Egr-1, Col4α1, MMP9, 3β-HSD4, and TGF-β2) were used. As observed in the menopausal study, Egr-1 mRNA abundance was increased in peri-OF diabetic mice compared with cycling diabetic mice (increase of 2.05-fold, P < 0.05). TGF-β2 was decreased in peri-OF diabetic mice compared with cycling diabetic mice (decrease of 1.6-fold, P < 0.05). In contrast to what was observed in post-OF diabetic mice, 3β-HSD4 was increased in peri-OF diabetic mice compared with cycling diabetic mice (increase of 1.2-fold, P < 0.05), whereas in the post-OF diabetic mice 3β-HSD4 was decreased compared with cycling diabetic mice.

**DISCUSSION**

VCD is a chemical by-product of rubber manufacturing which has been demonstrated to selectively destroy primordial and primary follicles in ovaries of rats and mice (reviewed in Ref. 8) without producing effects in large follicles or other tissues (20). We recently described the novel use of VCD as a means to induce gradual ovarian failure in mice (12). Ovarian failure in rodents is analogous to menopause in humans; thus VCD-treated mice serve as a new model of human menopause. Unlike previous rodent models of menopause, the VCD model preserves the period of irregular cycling and fluctuating hormone levels which precedes ovarian failure, termed perimenopause in humans (13). Also, following ovarian failure in the VCD model, the follicle-deplete ovaries secrete androgens, similar to the ovaries of postmenopausal humans (20).

**Post-ovarian failure diabetic mice.** In this study, we combined the VCD model of menopause with STZ-induced type 1 diabetes. Two groups of mice were studied: one in which diabetes was induced during impending ovarian failure (peri-menopause), and one in which diabetes was induced post-ovarian failure (menopause). Following the induction of diabetes blood glucose levels were significantly higher in post-ovarian failure diabetic mice than in cycling diabetic mice. Post-ovarian failure mice also had higher blood glucose than mice in which diabetes was induced during impending ovarian failure (peri-ovarian failure).

STZ causes diabetes by destroying insulin-producing pancreatic β-cells via oxidative stress-induced apoptosis. Recent data suggest that the presence of estrogen may protect β-cells from STZ-induced oxidative stress. In a study in which STZ was used to induce diabetes in ovariectomized rats, estradiol-treated ovariectomized rats had significantly lower blood glucose than those untreated with estradiol. The authors speculated that estrogen may attenuate STZ-induced destruction of pancreatic β-cells by decreasing inflammation (29) and thus may contribute to lower blood glucose levels in estrogen-treated rats. More recently, an in vitro study using STZ on primary cultures of pancreatic β-cells demonstrated that estrogen protects β-cells from oxidative stress-induced apoptosis (10).

Data from human studies suggest that estrogen also exerts a protective effect on pancreatic β-cells in humans. In the Women’s Health Initiative Hormone Trial, the incidence of newly diagnosed cases of diabetes was lower in women on hormone replacement therapy than in placebo-treated women (17). In perimenopausal women, longer cycle length (i.e., lower estrogen levels) has also been associated with higher blood glucose levels and hyperinsulinemia (19). Furthermore, in diabetic postmenopausal women, treatment with hormone replacement therapy improved insulin resistance (17).

**Cell proliferation in post-ovarian failure diabetic mice.** Renal cell proliferation and hypertrophy are early complications of diabetes (14, 24, 38); therefore, expression of PCNA can be used as a marker of early kidney damage. In our study,

![Blood glucose measurements in peri-ovarian failure diabetic mice.](http://ajprenal.physiology.org/)

Fig. 6. Blood glucose measurements in peri-ovarian failure diabetic mice. Mice were injected with STZ to induce diabetes, and fasted blood glucose was measured 6 wk after STZ treatment. Results are expressed as mean ± SD. Significant difference determined by Student-Newman-Keuls post hoc test following 1-way ANOVA. Filled bars, perimenopausal study; open bars, menopausal study. Data from menopausal study (Fig. 2) is shown for comparison. NS, nonsignificant.
expression of PCNA was increased in kidneys from post-ovarian failure diabetic mice compared with cycling diabetic mice. These data suggest that renal damage develops more rapidly and/or severely in post-ovarian failure diabetic mice compared with cycling diabetic mice.

Low estrogen levels increase susceptibility to nephropathy (5), thus the loss of estrogen in post-ovarian failure diabetic mice may have contributed to the increased proliferation observed in this study. However, high circulating blood glucose has been positively correlated with an increase in diabetic nephropathy in mice (6), thus the higher levels of glucose we observed in post-ovarian failure diabetic mice could have contributed to the increased cell proliferation in our study.

Changes in gene expression in post-ovarian failure diabetic mice. Diabetic kidney damage is usually associated with the increased accumulation of extracellular matrix proteins, such as collagen and fibronectin. This increase is thought to be stimulated by increased expression of TGF-β and the decreased expression and activity of matrix metalloproteinases (16, 32, 40). This study identified several genes which were differentially expressed in the renal cortex of post-ovarian failure diabetic mice and cycling diabetic mice. For example, the expression of 3β-HSD4 was significantly lower in post-ovarian failure diabetic mice than in cycling diabetic mice. MMP9 expression was decreased compared with cycling diabetic mice. This result correlates well with previous in vivo studies in which MMP9 protein expression and activity level were decreased in ovariectomized diabetic rats (16) and in ovariectomized Dahl salt-sensitive rats (18). Previous studies in mesangial cells have found that estrogen increases the expression of MMP9 (26); thus the loss of estrogen in our model of menopause may explain the observed decrease in MMP9 mRNA expression.

We observed a decrease in mRNA abundance of collagen 4α1 and TGF-β2 in post-ovarian failure diabetic kidneys compared with cycling diabetic kidneys. These data diverge from studies in which ovariectomy was associated with increased expression of TGF-β and collagen 4 in diabetic rats (15, 16).

mRNA abundance of 3β-HSD4 was significantly lower in the renal cortex of post-ovarian failure diabetic mice than in cycling diabetic mice. Murine 3β-HSD4 is a 3-ketosteroid reductase which metabolizes progesterone and dihydrotestosterone to their inactive forms (2, 27). Recent studies identified 3β-HSD4 expression as predictive of the degree to which glomerulosclerosis develops in the diabetic kidney, with lower expression of 3β-HSD4 correlating with more severe glomerulosclerosis (35). A decrease in 3β-HSD4 expression could lead to an increase in dihydrotestosterone levels. Dihydrotestosterone has been demonstrated to cause an increase in cell proliferation in a proximal tubule cell line (25); thus decreased 3β-HSD4 expression could result in increased proliferation of proximal tubule cells. Our results are consistent with this hypothesis as we found both a decrease in 3β-HSD4 mRNA abundance and an increase in cell proliferation in kidneys from post-ovarian failure diabetic mice compared with cycling diabetic mice and control mice.

Peri-ovarian failure diabetic mice. The VCD model of menopause is unique among rodent menopause models in that it retains residual ovarian tissue after ovarian failure and mimics the perimenopausal period. Recent studies find that changes in disease risk factors begin during perimenopause (7, 19) suggesting that the perimenopausal period may be critical in the development and prevention of estrogen-influenced diseases (37).

Data reported here found a trend toward increased glucose levels in peri-ovarian failure diabetic mice and a significant difference in blood glucose between post-ovarian failure diabetic mice and cycling diabetic mice. These data suggest a continuing trend of increasing blood glucose throughout the menopausal transition as estrogen gradually decreases.

Real-time PCR data from this study suggest that declining estrogen levels during peri-ovarian failure affect the expression of genes implicated in the development of diabetic kidney disease. For example, mRNA abundance of TGF-β was lower in peri-ovarian failure diabetic mice than in cycling diabetic mice. This is similar to the decrease in mRNA abundance for this gene observed between post-ovarian failure diabetic mice and cycling diabetic mice. These data highlight the importance of the VCD model of perimenopause and menopause, as these changes in gene expression may be important in understanding disease progression and have implications for disease prevention and treatment.

In conclusion, this study suggests that estrogen may play an important role in protecting the kidney from diabetes-induced cell proliferation and disease. It is possible that not all of the differences we observed between post-ovarian failure diabetic mice and cycling diabetic mice are due to changes in estrogen levels. The increase in blood glucose levels in post-ovarian failure diabetic mice may account for some of the differences in gene expression and cell proliferation we observed. Also, preliminary evidence suggests androgens negatively affect the development of kidney disease (22, 28). The VCD model of menopause preserves the androgen-producing function of the post-ovarian failure ovaries (20); thus some of the differences we observed between post-ovarian failure diabetic mice and cycling diabetic mice may be due to differences in androgen levels or differences in the estrogen/androgen ratio as opposed to direct actions of estrogen alone.

As more women develop diabetes at younger ages, the number of women with diabetes at the time of the menopausal transition will increase, as will the number of postmenopausal women with diabetes. Here, we introduce a new model for studying the impact of the menopausal transition on the development of diabetic kidney disease. We show that blood glucose levels are increased in post-ovarian failure diabetic mice compared with cycling diabetic mice and that there are differences in the mRNA abundance of genes associated with diabetic kidney disease in post-ovarian failure diabetic mice compared with cycling diabetic mice. Furthermore, we show there is increased cell proliferation in post-ovarian failure diabetic mice compared with cycling diabetic mice. Finally, we present data which suggest the importance of studying the development of diabetic kidney disease in the context of the perimenopausal period. Together, these data highlight the importance and utility of the VCD model of menopause, as it provides a physiologically relevant system for determining the impact of diabetes on the kidney during the menopausal transition.

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