Diabetes-Induced, Progressive Endometrial Involution
Characterization of Periluminal Epithelial Lipoatrophy

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The present studies detail the cytopathological alterations in uterine epithelial, basal lamina, and stromal endometrial subregions, and associated endocrine parameters that occur during the progressive exacerbation of the diabetes syndrome in this species of mouse. These alterations result in a cellular lipoatrophic condition that compromises uterine tissue integrity and promotes reproductive involution. Uterine tissue samples were obtained from litter-matched control (+/?)) and diabetic (db/db) C57BL/KsJ mice at four designated stages of the progressive expression of the diabetes mutation. In db/db mice between the ages of 4 and 12 weeks, the uterine epithelial cellular architecture exhibited progressive deterioration, characterized by cytoplasmic lipid inbibition (accumulation), organelle disintegration, apical membrane ciliary regression, and peristromal lamina separation from basal membrane surfaces, as compared with control indexes. The cytoplasmic volume occupied by lipid inclusions dominated the epithelial cells in diabetic mice, presenting dense basal pole lipid vacuoles, with perinuclear-intracytoplasmic migration of the inclusions promoting an apical cytoplasmic lipid condensation of increasing volume 8–12 weeks after mutation expression. These cytoplasmic lipid accumulations occurred under altered metabolic and endocrine conditions characterized by hyperglycemic, hyperinsulinemic, hypertriglyceridemic, and enhanced noradrenergic indexes, which were exacerbated between 4- and 12-week stages. These structural changes were accompanied by enhanced adrenergic counterregulatory metabolic responses as well as elevated lipoprotein and triacylglycerol lipase activities. These data indicate that diabetes-associated uterine involution is characterized by a progressive cellular and peristromal lipoatrophy of epithelial cell cytology and metabolic parameters, promoting stromal separation and ultimate endometrial involution.

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RESEARCH DESIGN AND METHODS

Animals. Adult female C57BL/KsJ mice (The Jackson Laboratory, Bar Harbor, ME) were used in these studies. Littermate control (+/+) and diabetic (db/db) genotypes were matched for phenotype, tissue sampling, blood glucose, and serum insulin concentration comparisons during the course of these studies. All mice were housed five per cage under controlled environmental conditions (23°C), with an established photoperiod of 12 h light/day (lights on at 0600 h).

Blood glucose levels (Ames Glucometer method) (8); serum insulin concentrations, as measured by radioimmunoassay analysis (Novo Industries, Copenhagen, Denmark; 17.2 μU/ml standards) (10); tissue neutral (NE) concentrations, as determined by high-performance liquid chromatography (HPLC) (12); tissue lipoprotein lipase activities (1,19); and body weights were monitored for each of the designated experimental periods (age 4 weeks, onset stage; age 8 weeks, progressive stage; age 12 weeks, mature overt stage; and age 16 weeks, tissue/organ regressive stage), as previously described (1). Animals exhibiting both obesity (≥ 20 g by age 4 weeks; controls ≤ 17 g) and pronounced hyperglycemia (≥ 200 mg/dl) relative to controls (≤ 150 mg/dl) were considered to be overtly diabetic (1,5), with the progressive expression of these indexes observed at ages 8–16 weeks (Fig. 1).

Tissue preparation and analysis. Uteri from control and db/db matched-paired groups ages 4, 8, 12, and 16 weeks were collected, weighed, and prepared for light and transmission electron microscopic (TEM) examination, as previously described (1–5). Tissue samples were sectioned and stained with toluidine blue for polychromatic lipid identification (1,3,20) and localization by light microscopic examination or with osmium tetroxide (4,5) before TEM examination. Tissue sections prepared for light microscopic analysis were used for cytoplasmonic polychromatic organelle designation, localization of intracellular lipid inclusion accumulations, and subsequent determination of the cytoplasmic volume changes that occur with progressive diabetes, as previously described (5). Photographic images of uterine epithelial cell populations from the prepared tissue samples were captured with an Olympus digital graphics camera and microscope unit (Olympus Optical, Tokyo, Japan), with lipid vacuole pools digitally enhanced using polychromatic stain identification and digital conversion to gray-scale imaging. The volume of the lipid-occupied epithelial cytoplasm was determined using a computer-assisted photodigital scanner. All cytoplasmic volume changes associated with diabetes-induced, progressive cellular lipoaerosity were expressed as the percent of total cellular volume occupied by lipid accumulations to comparable age-matched, littermate control tissue samples (i.e., control versus diabetic cytoplasmic lipid volume at each indicated stage of the expressed diabetes mutation).

Tissue norepinephrine, lipoprotein lipase, and triacylglycerol lipase measurements. To relate the recognized diabetes-induced changes in cellular and tissue structural indexes to concomitant alterations in cellular metabolic status, endometrial tissue samples were collected from control and diabetic groups ages 4, 8, and 16 weeks for analysis of noradrenergic and lipid uptake parameters. Homogenized, fresh tissue NE levels were determined by HPLC using a C-18 reverse phase ulHPLC pack column, as previously described and validated (12,13). Tissue lipoprotein and triacylglycerol lipase activities were measured as previously described (1) from triplicate
dexes that occur during the progressive, developmental expression of the diabetes-obesity syndrome, and their relation to systemic hyperglycemic and hyperinsulinemic, as well as hyperadrenergic and endocrine, indexes has not been reported. The present studies were undertaken to describe the cytostructural and associated endocrine alterations in uterine epithelial and peristromal tissues that occur during the stage-like progression of the diabetes-obesity syndrome, resulting in female reproductive dysfunction and tissue involution in this mutant murine model.

FIG. 1. Alterations in body weight, uterine weight, blood glucose, and serum insulin concentrations in control (+/+; white square) and diabetic (db/db; black square) C57BL/KsJ mice at ages 4, 8, 12, and 16 weeks. All values are means ± SE (five to nine animals per group). *P < 0.05.

FIG. 2. Photomicrographic (× 1000) analysis of cytoplasmic lipid vacuole accumulation and distribution patterns in 4- to 16-week-old control (+/+; C) and diabetic (db/db; D) groups (rows A–F) using light (column 1), digital photoluminescent intracellular lipid enhancement (column 2), and digital lipid subtraction (column 3) techniques for the determination of temporal and progressive volume alterations in diabetes-associated cytolipoatrophy of endometrial periluminal epithelial cells. The typical patterning of basal cytoplasmic lipid vacuole accumulations are presented from 4- (row A; C4) and 16-week-old (row B; C16) control group photomicrographs. Basal pole lipid (column 1: blue stain; column 2: fluorescent yellow) characterized subnuclear cytoplasmic regions in control epithelial cells during the age duration of the experiments and remained relatively constant in both volume and distribution patterns. In contrast, the enhanced basal pole lipid accumulations observed in the young (age 4 weeks) db/db endometrial epithelial samples (row C; D4) continued to increase in volume (rows D [D8] and E [D12]) in db/db groups ages 8 and 12 weeks. Perinuclear and supranuclear cytoplasmic distribution patterns were pronounced in all db/db samples relative to controls (C4, C16). By age 16 weeks (row F; D16, columns 1 and 2), pronounced epithelial cytoklypopathyemia was exhibited throughout the periluminal epithelial cell layer of diabetic endometrial tissue samples.
FIG. 3. Electron photomicrographs (×11.6–17.2 k magnification) of basal pole regions of endometrial epithelial cells from control (+/−; A) and diabetic (db/db; B) mice at age 8 weeks. Although cytoplasmic nuclear and basal membrane laminations are indicated by smooth surface layering supported by a homogenous basal lamina and stromal region in controls (A), the cytoarchitecture of diabetic (B) epithelial cells presented dramatic contrasts in shape and organization. Whereas dense accumulations of basal pole lipid vacuoles dominated the peristromal cytoplasmic region of endometrial epithelial cells, the basal membrane demonstrated an enhanced laminar folding pattern (open arrows) and spatial separation from the underlying stroma (C). Dense vacuole accumulations occurred in association with expressed hyperglycemia as well as elevated tissue lipoprotein lipase and triacylglycerol activities (Fig. 7) and accounted for the progressive enhancement of cytolipidemic volume measurements (Fig. 4).
pools of endometrial samples collected from 4-, 8-, and 16-week-old control and diabetic groups. All group mean (±SE) values were expressed as nanomoles of free fatty acid (FFA) per minute per gram of tissue.

**Statistical analysis.** Values for body weight, blood glucose, serum insulin levels, and tissue metabolic indexes were expressed as group means (±SE) for the designated age and genotype groups for both control and diabetic mice. Alterations in cytoplasmic volume measurements of intracellular lipid pools were expressed as individual volume per cell determinations from representative samples of each tissue examined. Intergroup differences with respect to age or genotype comparisons were determined using Student’s *t* test, Newman-Keuls, or ANOVA exams, where appropriate, with *P* ≤ 0.05 accepted as representing statistically significant intergroup measurement differences.

**RESULTS**

**Progressive changes in body and tissue weights and blood glucose and serum insulin indexes with expression of the db/db mutation.** At age 4 weeks (Fig. 1), the acute expression of the db/db mutation induced noticeable alterations in body phenotype, tissue indexes, and the endocrine-related parameters of C57BL/KsJ mice relative to controls. Increased body weights in db/db mice were related to concomitant elevations in blood glucose and serum insulin levels when compared with control values. These indexes contrasted with a dramatic decrease in uterine tissue weights in db/db groups relative to controls (Fig. 1). Both body and tissue weight indexes matured through the 8- to 12-week age period in controls, whereas blood glucose and serum insulin levels remained constant (Fig. 1). In contrast, all parameters remained elevated in the db/db groups through the same experimental period, with the exception of declining uterine tissue weights. The progressive exacerbation of these db/db mutation–induced indexes culminated with db/db littersmates that were approximately double the body weight, but which possessed 33% of the uterine tissue weights, of matched controls.

**Ultrastructural and cytomorphometric analysis of progressive endometrial epithelial lipoatrophy.** Epithelial basal-pole lipid accumulation was a common attribute of endometrial tissue samples examined by both light (Fig. 2) and electron microscopy (Fig. 3) at ages 4–16 weeks in control and diabetic groups. However, the cytoplasmic distribution, volume, and density accumulation of lipid inclusion vacuoles was progressively enhanced in db/db groups during the continued expression of the syndrome (Fig. 4). Control samples at ages 4–16 weeks demonstrated a consistent baso-polar distribution pattern of lipid inclusions that accounted for 3–18% cytoplasmic volume occupation (Fig. 4). In contrast, db/db epithelial cells at ages 4–16 weeks demonstrated a dramatic increase in cytoplasmic lipid volume (Figs. 2–4) and an expanded distribution pattern of cytoplasmic lipid inclusions (Figs. 1 and 2). A progressive increase in lipid absorption resulted in a 3–62% range of uterine epithelial cytoplasmic volume occupation by these compounds in db/db groups at ages 4–16 weeks (Fig. 4). The initial increases in basal lipid pools between 4 and 8 weeks in the db/db mice (Fig. 2, rows C and D) gradually expanded to include mid-cytoplasmic, perinuclear regions (Fig. 2, rows E and F) and apical cytoplasmic loci by 12–16 weeks. Enhanced cytoplasmic lipid volumes (Fig. 4) and diminished epithelial organelle presence (Fig. 3) characterized db/db cytoarchitecture.

Epithelial hypercytolipidemia was accompanied by dramatic alterations in db/db basal membrane and periluminal stromal structure (Figs. 3 and 5). Compared with controls (Fig. 3A), the basal membrane of db/db epithelial cells (Fig. 3B and C) demonstrated a folded contour along the peristromal borders (Fig. 5), the location where lipid accumulation and cytoplasmic inclusion clustering occurred. The basal region of db/db epithelial cells was transformed into adipocyte-like cell space, characterized by minimal organelle and enhanced membrane contouring, which promoted separation from the adjacent periluminal stroma (Fig. 3C). The dense lipid pool aggregations in the baso-polar regions were phase separated by apparent lipophobic/hydrophilic cytoplasmic fluids, which were eventually displaced, allowing for the progressive expansion of the inclusion volume and lipid inclusion size throughout the cytoplasm (Fig. 5). Separation between epithelial and periluminal stromal cell sites was progressively enhanced, and occurred in association with early indications of stromal cell isolation and atrophy, as well as diminished uterine tissue weight (Fig. 1).

**Endometrial adrenergic, triacylglycerol, and lipoprotein lipase response to progressive db/db-induced cytolipidemia.** Tissue NE levels were elevated by age 4 weeks in db/db mice relative to controls (Fig. 6), and remained elevated throughout the chronic expression of the db/db mutation, indicating that systemic counterregulatory responses to the hyperglycemic-hyperinsulinemic environment were invoked during the acute stages of progressive cytolipidemia-induced tissue involution. Concomitant elevations in tissue triacylglycerol and lipoprotein lipase activities (Fig. 7) indexed the nature of the hyperlipidemic state, and paralleled (evoked) the observed alterations in db/db tissue NE indexes. The lipoprotein lipase activity was markedly increased in db/db tissue samples during the course of these studies (Fig. 7) compared with corresponding control samples, suggesting that an associated decline in expressed cellular energy metabolism occurred in concert with progressive cytoplasmic hyperlipidemia and tissue involution.
FIG. 5. Electron photomicrographic (×25–35 k magnification) analysis of basal pole lipid accumulation and cytoplasmic perinuclear and apical migration of lipid vacuoles in endometrial periluminal epithelial cells of diabetic (db/db) C57BL/KsJ mice ages 8–16 weeks (A). Pronounced basal membrane folding, peristromal isolation and separation, and the intracytoplasmic dispersion of lipid vacuoles characterized the db/db tissue sample profiles. The progressive expansion of vacuole volumes (B) and density appeared as a migration of basal membrane stromal transmembrane accumulations, subjected to hydrophobic migration from the basal pole toward subnuclear sites, where lipophilic expansion occurred. The transmembrane movement of stromal lipid pools into basal cytoplasmic regions (C; closed arrows) along the epithelial peristromal surface in db/db tissues accounted for the progressive expansion of the cytoplasmic volume occupied by lipid vacuoles. Lipophilic attractions (C; open arrows) between adjacent vacuoles probably accounted for the massive basal pole accumulations before the intracytoplasmic traffic dispersion of vacuoles toward perinuclear and apical cytoplasmic regions.
progressive imbibition and accumulation of cytoplasmic lipid stored observed in the current studies correlates with the temporal expression of the hyperglycemic-hyperinsulinemic state and the enhanced tissue triglyceride levels present in endometrial samples of\( db/db \) groups relative to controls. In addition, the failure of enhanced tissue noradrenergic counterregulatory responses (12,13) to modulate the observed structural and metabolic indexes, coupled with stimulated lipoprotein lipase enzyme activity indexes, indicates that the progressive lipid insult on cellular integrity promotes or induces cellular lipoatrophy, which then generates endometrial cellular regression and subsequent reproductive tract involution. These results suggest that the reproductive decline characteristic of many type 2 diabetes models, including humans, may be related to the noted cellular lipoadiposity and metabolic alterations that occur after chronic exposure to the hyperglycemic-hyperinsulinemic environment and the inability of recognized counterregulatory systemic factors to correct these homeostatic insults to cellular function (1,3,12,13). Previous studies have indicated that some of the structural features characterized in the present studies may be stabilized in the\( db/db \) model by endocrine replacement therapy of the affected female reproductive tract parameters, if therapy is initiated before the early onset of the syndrome expression (5). The recognized lipolytic actions of administered estrogenic steroids (5) or etiocholanolones (22) may effectively maintain endometrial cellular integrity by reversing or suppressing the deleterious actions of chronic lipid inhibition on reproductive tract structure by normalizing intracellular homeostatic conditions and systemic counterregulatory, noradrenergic influences (13).

The observed baso-polar epithelial lipid accumulation that took place via enhanced basal membrane transport from stromal periluminal vasculature may have occurred as a result of the diabetes-associated compromise of endometrial blood flow perfusion rates (2,8,23). Utero-ovarian blood flow is diminished in diabetic subjects, inducing endometrial capillary vascular expansion with diminished flow exchange rates (2). Under such conditions, vasolipid deposition is enhanced (21), stromal metabolism and cellular integrity are compromised (1), and transmembrane lipid exchange is potentiated (4,13), resulting in a basal lamina saturation of triglyceride pools available for imbibition by endometrial epithelial cells (1). The result of such pericellular alterations in nutritional pools promotes the cytolipidemia-induced cellular atrophy observed in the luminal epithelium of the\( db/db \) endometrium in the present studies. The progressive accumulation of intracellular lipid inclusions compromises normal cellular triglyceride utilization and metabolic homeostasis associated with intracellular lipid mobilization, as evidenced by the alterations in lipase enzyme activities. The metabolic depression, or energy utilization shift, that consequentially occurs depresses cellular metabolic indexes, thereby promoting the transformation of functional epithelial cells into adipocyte-like lipid storage entities that are incapable of supporting epithelial or endometrial tissue functions (1). The resulting consequence of such a pattern of cellular structural and metabolic transformation is the noted compromise of reproductive tract functional integrity.

DISCUSSION
The progressive triglyceride accumulation and intracellular lipid distribution pattern, as well as the metabolic consequences of endometrial hypercytolipidemia associated with the chronic expression of the diabetes-obesity syndrome mutation in C57BL/KsJ mice, potentiates the expressed reproductive dysfunction and tissue atrophy in this model (1,5,12,13,15). The results of the present studies extend previous reports of reproductive tract atrophy, utero-ovarian cellular degeneration, and depressed ovarian steroidogenesis resulting from the chronic diabetes syndrome in various genetic and experimentally induced hyperglycemic models (1,2,5,8,13,15,21) by identifying the progressive nature of the hypercytolipidemia that characterizes this metabolic mutation. The evaluation of the
In summary, the recognized structural, endocrine, metabolic, tissue, and cytocellular indexes examined defined a progressive pattern of change that is exacerbated with the continued expression of the \textit{db/db} mutation and associated systemic conditions. Involvement of the reproductive tract, both structurally and functionally, is the apparent result of induced cellular transformation into adipocyte-like cells that are incapable of supporting normal reproductive tract parameters. The mechanisms that initiate these lipoatrophic changes, and the potential for the therapeutic modulation or suppression of the genetic mutation-induced alterations in cellular structural and metabolic indexes, are currently under investigation.

**REFERENCES**
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