Effect of diabetes mellitus during the luteal phase of the oestrous cycle on preovulatory follicular function, ovulation and gonadotrophins in gilts

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Development of preovulatory follicles was studied during the oestrous cycle in two experiments designed to examine the effects of short-term lack of insulin on preovulatory follicular function and (Expt 2 only) ovulation. In Expt 1, on day 12 of the third postpubertal oestrous cycle, insulin treatment was discontinued in streptozocin-induced diabetic gilts (n = 4), and on day 18, ovaries were removed from the diabetic gilts and from four normal untreated gilts. Diabetic gilts had a higher percentage of macroscopically atretic follicles (29.4 versus 6.8%; SEM = 5.9, P < 0.03) than did normal gilts. Binding of ¹²⁵I-labelled hCG by freshly collected granulosa cells from non-atretic follicles was similar in diabetic and normal gilts. Diabetic gilts had more LH peaks in 3 h on days 12–17 of the oestrous cycle than did normal gilts (2.3 versus 1.6; SEM = 0.12; P < 0.01). Serum oestradiol and progesterone concentrations were not affected by treatment, but serum testosterone was increased (P < 0.01) in diabetic gilts. In Expt 2, insulin treatment was withdrawn from nine diabetic gilts on day 12 of the oestrous cycle and ten normal gilts served as controls. On day 18, ovaries were removed from six diabetic and six normal gilts; four normal and three diabetic gilts were ovarioectomized 25 days after oestrus. Follicular diameter of diabetic gilts tended to be smaller than that of control (control: 3.95 versus diabetic: 3.01 mm; SEM = 0.4, P < 0.08) and the proportion of follicles with histologic evidence of atresia was higher in diabetic gilts (control: 29 versus diabetic: 47%; SEM = 5; P < 0.05) on day 18. In both experiments, the insulin-like growth factor I (IGF-I) and oestradiol concentrations of follicular fluid of diabetic gilts untreated with insulin from day 12 to day 18 was lower than in nondiabetic gilts. After day 18 in Expt 2, normal gilts exhibited oestrus (duration of cycle was 20 ± 0.5 days) accompanied by preovulatory surges in oestradiol and LH, whereas diabetic gilts did not exhibit oestrus or ovulate. In diabetic gilts, oestradiol concentrations were lower compared with those of normal gilts, and LH patterns were characterized by two (two gilts) or three (one gilt) increases of more than 2 ng ml⁻¹ between day 18 and day 25. Thus, impaired follicular function in diabetic gilts is not explained by decreased function of the hypothalamo–pituitary axis, since LH was not decreased. We conclude that the decreased oestradiol production by diabetic gilts indicates either disrupted steroidogenesis due to the acute absence of insulin or lowered IGF-I, or both.

Introduction

In pigs, ovarian follicles enter ovariolytic or degenerative pathways between day 14 and day 18 of the oestrous cycle (Clark et al., 1982; Foxcroft and Hunter, 1985). We reported that administration of insulin during this time increased ovulation rate (Cox et al., 1987) and decreased macroscopic signs of follicular atresia in cyclic pigs and in pigs treated with pregnant mares’ serum gonadotrophin (PMSG) (Matamoros et al., 1990, 1991). Untreated diabetes in pigs, caused by withdrawal of insulin for 4 days of follicular growth induced by PMSG, increased atresia of small follicles but did not affect the number of presumed preovulatory follicles (Meurer et al., 1991). However, in that study, concentrations of oestradiol and insulin-like growth factor-I (IGF-I) were lower in large (>7 mm diameter) follicles of diabetic gilts than in those of non-diabetic gilts, indicating abnormal hormone secretion despite similar numbers of follicles. Concentrations of IGF-I were also lower in
peripheral serum, but intrafollicular steroidal differences were not reflected in peripheral serum.

The inhibitory influences of diabetes on follicular function have been attributed to various portions of the hypothalamo–hypophyseal–ovarian axis, and all three parts of the axis may be affected under different conditions (Kirchick et al., 1978; Djursing et al., 1982; Katayama et al., 1984; Valdes et al., 1991). However, there is evidence that complex paracrine control, involving growth factors and metabolic hormones, could account for follicular development in the absence of changes in gonadotrophins (see for example, Tonetta and diZerega, 1989). Meurer et al. (1991) did not observe any negative effect of diabetes on circulating gonadotrophins in PMSG-treated gilts; in fact, in two gilts LH concentrations increased 72 h after PMSG. However, PMSG-induced follicular growth is known to be associated with low LH and FSH concentrations (Flowers et al., 1989). Furthermore, follicular growth induced by exogenous gonadotrophins exhibits distinct functional differences from that in cyclic gilts (Wiesak et al., 1990). The major objective of the present experiments was therefore to evaluate the effects of insulin withdrawal on preovulatory follicular growth (Expts 1 and 2), ovulation (Expt 2), and associated gonadotrophin concentrations (both Expts) in cyclic gilts. A secondary objective was to determine whether intrafollicular influences of diabetes on steroids and IGF-I were reflected in secretion of hormones into the ovarian venous circulation (Expt 1).

Materials and Methods

Experiment 1

Eight crossbred (Yorkshire × Duroc) gilts that had been unilaterally ovariectomized before puberty as part of a previous study (Meurer et al., 1991) were used during their third postpubertal oestrous cycle. Four gilts were non-diabetic (144 ± 6 kg body mass at the time of this study) and four were streptozocin-induced diabetic (139 ± 6 kg body mass). Briefly, diabetes was induced between 8 and 12 weeks of age, and gilts were maintained on continuous insulin therapy until the present experiment, except for a period of 4 days after the induction of follicular development with PMSG at 151 ± 4 days of age as part of the previous experiment (Meurer et al., 1991). Age at puberty was 182 ± 3 days for control gilts and 187 ± 4 days for diabetic gilts. Feed intake of normoglycaemic gilts during the entire period after induction of diabetes was limited to approximately 90% of maximum intake to keep body masses (determined weekly) similar to the diabetic animals, which were fed ad libitum as described by Meurer et al. (1991). During the experimental period, normal gilts remained on the feed intake calculated to maintain their masses similar to those of diabetic gilts. Insulin therapy maintained plasma glucose at similar concentrations to that of non-diabetic animals, between 80 and 100 mg per 100 ml during the pre-experimental period as previously described, and the existence of diabetes was confirmed by a glucose challenge test before death (Meurer et al., 1991).

All pigs were catheterized before 13:00 h on day 11 of the third oestrous cycle (first day of oestrus = day 1) with a polyethylene catheter placed nonsurgically and aseptically into the anterior vena cava. Day 12 was the first day without insulin therapy. Blood samples were obtained every 12 h from 18:00 h on day 11 to 06:00 h on day 18 of the oestrous cycle and for a 3 h period on each of days 12–17 for analysis of LH pulses (samples every 15 min between 10:00 and 13:00 h).

On day 18, ovaries were removed from each gilt under anaesthesia induced with sodium pentothal (Biotal, 3 mg kg⁻¹ i.v.; Boehringer-Ingelheim Animal Health, Inc., St Joseph, MO) and maintained with methoxyflurane (Pitman-Moore Inc., Washington Crossing, NJ) and nitrous oxide. A blood sample from an ovarian vein was obtained approximately 1 cm from the ovary immediately before removal of the ovary on day 18. Immediately after removal of the ovary, the diameters of all visible follicles were measured to the nearest millimetre and follicles were assigned to a size class (small: < 3 mm; medium: 3–6 mm or large: ≥ 7 mm). Macroscopic signs of atresia were noted following the procedures of Moor et al. (1978) and Matamoros et al. (1990). Fluid was aspirated from individual non-atretic follicles and diluted in 0.01 mol PBS I⁻¹ containing 0.1% gelatin at pH 7.5 to dilutions ranging from 1:10 to 1:40, depending on the volume of follicular fluid available and the amount needed for radioimmunoassays. Macromorphically atretic follicles were excluded because fluid viscosity prevented reliable collection. The diluted follicular fluid was then frozen until assays were performed. Granulosa cells from up to four (if available) macroscopically non-atretic follicles from each size class, for a total of 73 follicles, were collected and assayed immediately for hCG binding according to the procedures of Matamoros et al. (1990). A total of 185 follicles was analysed for oestradiol, progesterone, testosterone and IGF-I.

Experiment 2

Ten contemporary non-diabetic and nine streptozocin-induced diabetic crossbred (Yorkshire × Duroc) gilts were used. Diabetes was induced at 118 ± 4.3 days of age and gilts were maintained on exogenous insulin treatment as in Expt 1. The later age was chosen, on the basis of observations in Expt 1 that puberty age, ovulation rate and cycle duration were not affected by diabetes mellitus, and the different periods of diabetes mellitus with insulin treatment were not expected to affect the results. Puberty onset in Expt 2 was at 185 ± 4.6 and 184 ± 4.9 days of age for normal and diabetic gilts, respectively. On day 12 of the second or third oestrous cycle (two normal and two diabetic gilts were on the third), insulin treatment was withdrawn from diabetic gilts. Ovaries were removed from six normal and six diabetic gilts on day 18 as in Expt 1. For the remaining gilts, i.v. catheters were inserted on day 18 as described for Expt 1 and blood samples were collected three times a day. Ovaries were removed and collection of blood samples was stopped 25 days after oestrus (designated this way because no diabetic gilts exhibited oestrus). Gilts were weighed on day 11, 17 and before surgery on day 18.

After the ovaries were removed, the size of all follicles was measured. One ovary had the follicular fluid removed for assay, as in Expt 1, and the second ovary was processed for histological assessment of atresia. For histology studies, the
ovaries were placed in Bouin’s fixative for 36 h, then prepared in paraffin wax sections (5 μm) and stained with haematoxylin and eosin (Carson et al., 1979). An atretic follicle was defined as one containing at least 10% pyknotic granulosa cells (five sections of 100 cells examined per follicle). The percentage of atretic follicles was based on observations of all follicles ≥3 mm diameter. Follicular development on day 18 was less advanced than it was in Expt 1; follicle size classes were therefore expressed as < 3, 3-6 and > 6 mm. Ninety-seven follicles were analysed for follicular fluid hormones and 73 for histologic evidence of atresia.

Assays

Follicular fluid was used at the initial dilutions described above for IGF-I, diluted to final concentrations of 1:100 or 1:200 in PBS for progesterone and testosterone and to 1:1000 for oestradiol radioimmunoassays, on the basis of preliminary determinations of dilution rates. Sample volumes assayed for steroids were as follows: 200 μl for dilutions from medium-sized and large-sized follicles and 400 μl or 600 μl for dilutions of small-sized follicles. Ovarian venous samples were diluted 1:200 for steroid radioimmunoassays. Over the dilutions used, validity of assays was not affected.

Serum LH concentrations were determined by a double-antibody radioimmunoassay using a rabbit anti-porcine LH serum (No. 566; provided by G. D. Niswender, Colorado State University, Fort Collins, CO) and porcine standard (LER 786-3; provided by L. E. Reichert, Albany Medical College, Albany, NY) validated previously (Niswender et al., 1970; Cox et al., 1987). Intra- and interassay coefficients of variation were 8.9 and 8.3%, respectively. Sensitivity of the assay was 0.12 ng ml⁻¹.

Serum FSH was assayed by validated procedures (Expt 1 only) using USDA-FSH-ppl as standard and USDA 1010-10 as antibody (both provided by D. G. Bolt, National Hormone and Pituitary Program, Beltsville, MD; USDA: Cox et al., 1987). Intra- and interassay coefficients of variation were 6.4% and 11.2%, respectively. Sensitivity of the assay was 0.3 ng ml⁻¹.

Concentrations of IGF-I in peripheral serum, follicular fluid, and ovarian venous serum were assayed according to validated procedures (Houseknecht et al., 1988; Matamoros et al., 1991) with the exception that 48 h after adding the tracer hormone, 100 μl of 4% normal rabbit serum in assay buffer and 200 μl of second antibody (1:10 in assay buffer) were added, followed by 1.0 ml ice-cold 6% polyethylene glycol (P2139, Sigma Chemical Co., St Louis, MO) in distilled water to aid in precipitation of antibody-bound hormone. Intra- and interassay coefficients of variation were 3.4 and 4.0%, respectively, and sensitivity of the assay was 1.9 ng ml⁻¹.

Concentrations of oestradiol in peripheral serum, follicular fluid, and ovarian venous serum were assayed by procedures validated in our laboratory for serum (Cox et al., 1988) and follicular fluid (Matamoros et al., 1990). Intra- and interassay coefficients of variation were 7.2 and 11.5%, respectively. Sensitivity of the assay was 1.6 pg ml⁻¹. Efficiencies of a single extraction in ethyl acetate were 95% in follicular fluid and 87% in serum, and values were corrected accordingly.

Concentrations of testosterone (Expt 1 only) in peripheral serum, follicular fluid and ovarian venous serum were assayed by procedures validated in our laboratory (Matamoros et al., 1990), with the exception that 125I-labelled testosterone (testosterone-3(0-carboxymethyl) oximino-2-[125I]iododihistine) (Amersham Corporation, Arlington Heights, IL) was used as the radioactive tracer. A single extraction with [³H]testosterone yielded efficiencies of 77% for follicular fluid and 85% for serum, and samples were corrected for extraction efficiency. Intra- and interassay coefficients of variation were 12 and 22%, respectively. Sensitivity of the assay was 20 pg ml⁻¹.

Progesterone in peripheral serum, follicular fluid, and ovarian venous serum (Expt 1 only) was assayed by procedures validated in our laboratory (Rainey et al., 1990) and modified for 125I-labelled progesterone (Meurer et al., 1991). Intra- and interassay coefficients of variation were 11.7 and 9.5%, respectively. Sensitivity of the assay was 0.03 ng ml⁻¹. A single extraction yielded recoveries of 72% for follicular fluid and 82% for serum, and samples were corrected for extraction efficiency.

Binding of hCG by freshly collected granulosa cells was assessed in Expt 1 as described by Matamoros et al. (1990). Plasma glucose concentrations were measured by the glucose oxidase-peroxidase method (Cox et al., 1987) and serum non-esterified fatty acids (Expt 1) as described previously (Matamoros et al., 1991).

Statistical analyses

Proportions of follicles in individual size classes were expressed as the percentages of total follicles present, such that there was a single observation for each animal. Proportions and numbers of follicles were analysed using one-way analysis of variance (Steel and Torrie, 1980) with treatment as the independent variable. For steroid concentrations and ratios in follicular fluid, models included effects of treatment, pig within treatment (error term used to test effect of treatment), follicle size class (< 3 mm, 3-6 mm and > 7 mm) and the interaction of size class and treatment. For circulating hormone concentrations, the model used included effects of treatment, pig within treatment (error term used to test effect of treatment), time and the time by treatment interaction. Where main effects and interactions were significant, means were separated by the method of least significant difference (SAS, 1988).

The methods of Goodman and Karsch (1980) were used to identify LH pulses. Variables obtained using these criteria were analysed for effects due to treatment using one-way analysis of variance (SAS, 1988).

Results

Experiment 1

During the period when diabetic gilts were without insulin, changes in mass of diabetic (− 2.3 ± 1.5 kg) and normoglycaemic gilts (− 1.5 ± 1.5 kg) were similar. On day 18, the numbers of corpora lutea were similar for non-diabetic and diabetic gilts (13.0 and 14.5, respectively; SEM = 1.5).
Table 1. Numbers of follicles and percentages of total follicles in each follicle class in non-diabetic control and diabetic gilts according to follicular size and macroscopic atresia (Expt 1)

<table>
<thead>
<tr>
<th>Class of follicle</th>
<th>Number of follicles</th>
<th>SEM</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
</tr>
<tr>
<td>&lt; 3 mm</td>
<td>11.8</td>
<td>21.3</td>
<td>9.2</td>
</tr>
<tr>
<td>3–6 mm</td>
<td>37.5</td>
<td>23.0</td>
<td>9.4</td>
</tr>
<tr>
<td>≥ 7 mm</td>
<td>5.8</td>
<td>3.8</td>
<td>3.0</td>
</tr>
<tr>
<td>All ≥ 3 mm</td>
<td>43.3</td>
<td>26.8</td>
<td>7.1</td>
</tr>
<tr>
<td>Atretic</td>
<td>4.3b</td>
<td>18.3c</td>
<td>3.9</td>
</tr>
<tr>
<td>Total</td>
<td>59.4</td>
<td>66.4</td>
<td>14.0</td>
</tr>
</tbody>
</table>

*Values are least squares means and SEMs are pooled estimates of variance.

Within a row, variable means with different superscripts are significantly different (P < 0.05).

Table 2. Intrafollicular hormones from each follicle size class in non-diabetic control and diabetic gilts (Expt 1)

<table>
<thead>
<tr>
<th>Size of follicle</th>
<th>State</th>
<th>Oestradiol* (ng ml⁻¹)</th>
<th>Progesterone (ng ml⁻¹)</th>
<th>Testosterone (ng ml⁻¹)</th>
<th>IGF-1 (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3 mm</td>
<td>Normal</td>
<td>7 ± 9</td>
<td>176 ± 86</td>
<td>37 ± 13</td>
<td>117 ± 15b</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>7 ± 6</td>
<td>111 ± 73</td>
<td>8 ± 11</td>
<td>68 ± 11c</td>
</tr>
<tr>
<td>3–6 mm</td>
<td>Normal</td>
<td>19 ± 3</td>
<td>188 ± 21</td>
<td>20 ± 4</td>
<td>115 ± 5b</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>17 ± 5</td>
<td>166 ± 31</td>
<td>12 ± 5</td>
<td>63 ± 8c</td>
</tr>
<tr>
<td>≥ 7 mm</td>
<td>Normal</td>
<td>95 ± 6b</td>
<td>185 ± 38b</td>
<td>32 ± 6</td>
<td>142 ± 9b</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>31 ± 6c</td>
<td>475 ± 39c</td>
<td>22 ± 7</td>
<td>76 ± 10c</td>
</tr>
</tbody>
</table>

*Values are least squares means and SEMs are pooled estimates of variance.

Within a column and size class, values with different superscripts are significantly different (P < 0.0001).

The diabetic state was confirmed by significantly higher plasma glucose in diabetic than in non-diabetic gilts throughout the sampling period (treatment by time interaction, P < 0.0001). On the first day without insulin treatment, plasma glucose concentration averaged 293 ± 9 mg per 100 ml in diabetic gilts. Over all sampling times, glucose averaged 447 and 83 mg per 100 ml in diabetic and non-diabetic gilts, respectively (SEM = 25). Serum non-esterified fatty acids increased in diabetic gilts over the 7 day period (treatment by time interaction, P < 0.0003), averaging 0.75 mEq l⁻¹ and 0.11 mEq l⁻¹ for diabetic and non-diabetic gilts, respectively (SEM = 0.17).

Diabetic gilts had similar numbers of macroscopically non-atretic follicles in each size class as did normal gilts, but a smaller percentage of total follicles present were ≥ 3 mm in diameter (Table 1). Diabetic gilts had higher numbers and percentages of atretic follicles than did control gilts. The highest incidence of atresia was in the small follicle size class and was greater for diabetic gilts (46 versus 10% of total small follicles; SEM = 7, P < 0.009, result not shown). Percentages of macroscopically atretic medium-sized follicles were 8 and 14% (SEM = 5) for normal and diabetic gilts, respectively, and were similar. There were no large atretic follicles in normal gilts, and only one diabetic gilt had large atretic follicles (n = 2).

Granulosa cell hCG binding was not affected significantly by treatment, averaging 92 and 63 ng per 10⁶ cells (SEM = 31) for all follicles of normal and diabetic gilts, respectively. Size class significantly (P < 0.05) affected binding of hCG: that of large follicles (153.9 ± 32.5 ng per 10⁶ cells) exceeding that of medium-sized (34.9 ± 27.1 ng per 10⁶ cells) and small follicles (33.0 ± 35.9 ng per 10⁶ cells).

In the two size categories of follicles < 7 mm, the diabetic state did not alter intrafollicular concentrations of oestradiol, progesterone or testosterone. However, in follicles ≥ 7 mm, diabetic gilts had significantly higher concentrations of progesterone and significantly lower concentrations of oestradiol than did controls. Diabetic gilts also had significantly lower concentrations of IGF-1 in all sizes of follicles than did controls (Table 2).

Ratios of oestradiol to other steroids were lower in large follicles of diabetic compared with non-diabetic gilts (oestradiol:testosterone, 2.06 ± 0.41 versus 4.78 ± 0.39; and oestradiol:progesterone, 0.09 ± 0.06 versus 0.73 ± 0.06; P < 0.05). For small and medium-sized follicles, ratios of oestradiol:progesterone were affected by diameter (0.54 ± 0.43 for small and 1.33 ± 0.26 for medium-sized follicles; P < 0.05), but not by treatment. Ratios of oestradiol:progesterone in small and medium-sized follicles averaged 0.14 ± 0.05 and were not affected by diameter or treatment.
Serum concentrations of IGF-I were similar for both treatments at the beginning of sampling but gradually declined in diabetic gilts after removal of insulin therapy (treatment by time interaction, \( P < 0.0001 \); Fig. 1).

When samples taken at 12 h intervals only were considered, temporal patterns of LH and FSH were not influenced by treatment, although FSH decreased with day \(( P < 0.05 \); Fig. 2\). The low LH concentrations evident in all gilts at 06:00 h on day 12 of the oestrous cycle cannot be explained. Any stress associated with the catheterization procedure the day before would not be expected to cause decreased gonadotrophin secretion (Diekman et al., 1990). Interference owing to processing of blood samples was ruled out, as gilts reached day 12 of the cycle on different days. In addition, all blood samples from this period were not contiguous in the radioimmunoassay for LH, and a repeat assay on these samples produced the same results.

Numbers of peaks of LH during 3 h sampling periods were greater for diabetic gilts (2.3 versus 1.6 peaks per 3 h, \( \text{SEM} = 0.12, P < 0.01 \)). The number of peaks also increased with day of the cycle \(( P < 0.01 \); Fig. 3\). Mean LH peak amplitude \((1.3 \pm 0.3 \text{ ng ml}^{-1})\), overall mean \((0.7 \pm 0.1 \text{ ng ml}^{-1})\), and mean baseline concentration \((0.4 \pm 0.1 \text{ ng ml}^{-1})\) were similar for diabetic and non-diabetic gilts.

There was no effect of treatment on concentrations of either progesterone or oestradiol in peripheral serum. Diabetic and normal gilts averaged 16.1 and 19.5 \text{ ng ml}^{-1} (\text{SEM} = 5) for progesterone and 4.6 and 3.8 \text{ pg ml}^{-1} (\text{SEM} = 1) for oestradiol, respectively. Serum testosterone increased after removal of insulin treatment (treatment by time interaction, \( P < 0.02 \); Fig. 4). Over all sampling times, testosterone in serum of diabetic gilts averaged 0.07 \text{ ng ml}^{-1} and in non-diabetic gilts 0.02 \text{ ng ml}^{-1} (\text{SEM} = 0.01).

In ovarian vein serum on day 18, concentrations of oestradiol \((0.20 \pm 0.10 \text{ ng ml}^{-1}, \text{SEM} = 0.09)\), testosterone \((0.21 \text{ and } 0.14 \text{ ng ml}^{-1}, \text{SEM} = 0.09)\) and progesterone \((22.5 \text{ and } 19.2 \text{ ng ml}^{-1}, \text{SEM} = 5)\) in non-diabetic and diabetic gilts, respectively, were similar. Diabetic gilts had lower IGF-I concentrations in ovarian vein serum than did normal gilts (36 and 90 \text{ ng ml}^{-1}, \text{SEM} = 4, P < 0.05) on day 18. Ovarian vein and peripheral concentrations of IGF-I were similar.

**Experiment 2**

On day 18, diabetic gilts had higher blood glucose concentrations compared with normal animals, confirming the diabetic state \((472 \text{ versus } 73 \text{ mg in } 100 \text{ ml}; \text{SEM} = 25; P < 0.001)\). From day 12 to day 18, diabetic gilts lost body mass while
Fig. 4. Least squares means of serum testosterone (SEM = 0.01) in samples obtained every 12 h during days 11–18 of the oestrous cycle for (− Δ − ) diabetic and (− O − ) normal gilts. Effects of treatment were significant (P < 0.05).

non-diabetic gilts maintained body mass (− 6.4 versus 0.0 kg; SEM = 1.7; P < 0.05). For the seven animals sampled until day 25, diabetic gilts lost body mass while non-diabetic gilts gained (− 7.7 versus 3.4 kg from day 12 until 25; SEM = 2.0; P < 0.001). The numbers of corpora lutea on day 18 were similar for diabetic and normal gilts (15.1 and 15.6, respectively; SEM = 2.0).

Numbers of medium-sized follicles (4–6 mm) tended to be lower in diabetic than in normal animals, whereas small follicles (< 3 mm) tended to be higher. The average diameter of follicles of normal gilts tended to exceed that of diabetic gilts (Table 3). Only two follicles from diabetic and four follicles from normal gilts were > 6 mm; hormonal data are therefore presented for the smaller size classes only. The percentage of histologically atretic follicles was higher in diabetic than in non-diabetic gilts. Despite the moderate effects on follicular size distribution compared with Expt 1, diabetes significantly decreased follicular fluid concentrations of IGF-I and oestradiol (Table 4). Peripheral concentrations of immunoreactive IGF-I were lower (P < 0.05) in diabetic gilts, and they did not change at oestrus in non-diabetic gilts (Fig. 5).

Of the group of gilts that were given the opportunity to exhibit oestrus, all non-diabetic gilts and no diabetic gilts were observed in oestrus. Concentrations of LH on day 18 were similar for normal and diabetic gilts (1.5 ± 0.3 ng ml⁻¹). However, normal gilts had preovulatory surges (peak magnitude 4.4 ± 0.3 ng ml⁻¹) that were preceded by sustained increases in oestradiol concomitant with behavioural oestrus (Fig. 6). The duration of the oestrous cycle for non-diabetic animals was 20 ± 0.5 days, and number of corpora lutea 5 days later was 16.3 ± 0.5. In contrast, diabetic gilts did not exhibit oestrus, did not ovulate and did not have a preovulatory oestradiol surge. On day 25, no corpora lutea and no follicles greater than 2 mm diameter were visible in diabetic animals. However, diabetic gilts had two (n = 2; Fig. 7a, c) or three (n = 1; Fig. 7b) sustained increases in LH which were smaller in peak magnitude (2.9 ± 0.3 ng ml⁻¹; P < 0.05) than preovulatory surges in non-diabetic gilts, but larger than LH concentrations on day 18 (P < 0.05). One diabetic gilt (Fig. 7b) had a brief increase in oestradiol on day 19 that was not sustained.

Peak oestradiol concentrations were 19 and 52 pg ml⁻¹ (SEM = 6; P < 0.01) for diabetic and non-diabetic gilts, respectively.

Discussion

Follicular development and the number of corpora lutea in Expt 1 indicate that the single ovaries had undergone compensatory growth and that insulin-replacement treatment supported a normal ovulation rate in diabetic gilts. In both Expts, similarity in age at puberty and number of corpora lutea between diabetic and normal gilts indicates that insulin-replacement treatment before the experimental removal of insulin was sufficient for normal ovarian function. The adequacy of our insulin-replacement therapy has been confirmed in another study in which follicular size distribution, incidence of atresia, follicular hormone concentrations and ovulation rate were similar in non-diabetic gilts and in gilts receiving insulin treatment (Howell et al., 1992). Follicles collected on day 18 in normal and diabetic gilts were less developed in Expt 2 than in Expt 1, but this difference is attributed to natural variation in follicular development rather than to any difference in experimental methods.

In Expt 1, acute onset of untreated diabetes decreased the proportion of follicles ≥3 mm, which are presumed to contain the ovulatory population (Clark et al., 1982). Follicular development in Expt 2 appeared to be at a less advanced stage overall than in Expt 1, although atresia was increased comparably with Expt 1. These results are consistent with earlier work in which the presence of insulin was negatively associated with follicular atresia (Matamoros et al., 1990, 1991; Meurer et al., 1991). In Expt 1, as well as in our previous study (Meurer et al., 1991), the greatest macroscopic atresia was in the population of small follicles, which undergo the most rapid atresia during preovulatory follicular growth (Dailey et al., 1975; Clark et al., 1982; Guthrie et al., 1990). Follicular size distribution was affected differently during the natural cycle than previously reported for PMSG-induced follicular growth (Meurer et al., 1991). In that study, only proportions of small...
(<3 mm) follicles were significantly affected by diabetes. In both studies, there were no negative influences of diabetes on gonadotrophin concentrations. However, differences in gonadotrophin stimuli to follicles (endogenous versus exogenous) may explain why different size populations of follicles were inhibited by diabetes during natural and PMSG-induced follicular growth. Wiesak et al. (1990) demonstrated differences in follicular size distribution and increased variation of follicular development in PMSG-induced compared with natural follicular growth.

The results of Expt 1 support the earlier postulation that the effect of diabetes on steroidogenesis either inhibits the conversion of progesterone to testosterone or the aromatization of testosterone to oestradiol (Meurer et al., 1991). The steroid differences occurred in follicles that appeared healthy; however, biochemical indications of decreased steroid production precede macroscopic signs of atresia (Moor et al., 1978; Terranova, 1979). The lowered ratios of oestradiol to progesterone and to testosterone in follicles ≥7 mm also indicate that steroidogenesis is most impaired in the largest follicles. Insulin has been demonstrated to affect ovarian transport of glucose (Allen et al., 1981; Otani et al., 1985). However, as diabetes affected progesterone and oestradiol in different ways, increasing the former and decreasing the latter, it is likely that the mechanism involves only certain aspects of steroidogenesis, rather than a general deficiency of energy substrate. The results of Expt 2 confirm the suppressive effect of diabetes on intrafollicular oestradiol.

Although there were no influences of diabetes on ovarian vein concentrations of steroids, peripheral testosterone was increased in diabetic gilts. If the ovary is the source of excess androgen production, as in hyperandrogenic obese women (Friedman and Kim, 1985), increased follicular fluid and ovarian vein concentrations of androgens would be expected, but these were not observed. In ovariectomized pigs, diabetes was also associated with high testosterone concentrations compared with those of non-diabetic gilts (0.06 versus 0.01 ng ml⁻¹, SEM = 0.03; C. A. Carlton and N. M. Cox, unpublished). Those values are similar to the values reported here in ovary-intact gilts and suggest an adrenal origin for the increased testosterone. In ovariectomized streptozotocin-diabetic rats, the adrenals have been implicated as a source of testosterone (Leaming et al., 1982).

In Expt 1, although steroid concentrations were similar in diabetic and non-diabetic gilts except when diameters were ≥7 mm, IGF-I was significantly lower in all sizes of follicles of diabetic gilts compared with those of non-diabetic gilts. In Expt 2 the small number of large-sized follicles prevents a direct comparison, but IGF-I was low in the follicles available from diabetic gilts. In our previous study (Meurer et al., 1991), IGF-I concentration was low only in the largest follicles of diabetic gilts stimulated with PMSG, again reflecting a possible difference between exogenous and endogenous gonadotrophin support. It has also been demonstrated that PMSG stimulates intrafollicular IGF-I in vivo (Hammond et al., 1988). Insulin and IGF-I share many actions on granulosa cell function, including

### Table 3. Number of follicles, diameter and histologic assessment of atresia for non-diabetic control and diabetic gilts (Expt 2)

<table>
<thead>
<tr>
<th>Follies</th>
<th>Normal</th>
<th>Diabetic</th>
<th>SEM*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number ≤3 mm</td>
<td>35.0</td>
<td>50.8</td>
<td>12.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Number 4–6 mm</td>
<td>32.3</td>
<td>21.6</td>
<td>5.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Number &gt; 6 mm</td>
<td>2.2</td>
<td>0.3</td>
<td>1.1</td>
<td>0.26</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>3.95</td>
<td>3.01</td>
<td>0.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Percentage atretic</td>
<td>29.0</td>
<td>47.0</td>
<td>5.0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Values are least squares means and SEMs are pooled estimates of variance.

### Table 4. Intrafollicular concentrations of immunoreactive insulin-like growth factor-I (IGF-I) and oestradiol on day 18 for non-diabetic control and diabetic gilts (Expt 2)

<table>
<thead>
<tr>
<th>Hormone (ng ml⁻¹)</th>
<th>Diameter of follicle</th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>≤3 mm</td>
<td>145 ± 16</td>
<td>71 ± 23</td>
</tr>
<tr>
<td></td>
<td>4–6 mm</td>
<td>153 ± 9</td>
<td>80 ± 12</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>≤3 mm</td>
<td>51 ± 11</td>
<td>17 ± 15</td>
</tr>
<tr>
<td></td>
<td>4–6 mm</td>
<td>79 ± 7</td>
<td>23 ± 8</td>
</tr>
</tbody>
</table>

*Values are least squares means and SEMs are pooled estimates of variance.

*bEffect of treatment (P < 0.05); †effect of treatment (P < 0.05) and diameter (P < 0.05).
The similarities in IGF-I concentrations among follicular fluid and venous sources does not support the notion of a significant ovarian contribution to peripheral IGF-I. In addition, in contrast to the work of Spicer et al. (1993) in sheep, IGF-I concentrations were not increased at oestrus in Expt 2. A similarity in peripheral and follicular fluid IGF-I concentrations has been observed in pigs (Hammond et al., 1988) and cattle (Echternkamp et al., 1990). However, it has been demonstrated that granulosa cells produce IGF-I in pigs (Hammond et al., 1985) and rats (Oliver et al., 1989; Harty et al., 1992). Diabetes mellitus decreased mRNA for IGF-I when examined in non-reproductive tissue from rats (Bornfeldt et al., 1989) and pigs (Leaman et al., 1990), so it is possible that insulin modulates granulosa cell IGF-I synthesis in the same way. Modulation of IGF-I and its binding proteins is associated with gonadotrophin-stimulated follicular development of pigs (Mondschein et al., 1990; Samaras et al., 1992).

The influences of diabetes on intrafollicular production of hormones could not be attributed to differences in ovarian receptors for LH. This result is similar to observations of normal gilts given exogenous insulin, in which there were no influences of exogenous insulin on hCG receptors of granulosa cells (Matamoros et al., 1990).

Although the diabetes did not exert sustained effects on overall concentrations of gonadotrophins, pulsatile LH secretion was increased during the preovulatory period in Expt 1. These results contrast with those for diabetic rats, in which preovulatory LH concentrations were decreased, ovulation was prevented (Kirchick et al., 1978, 1982; Katayama et al., 1984) and LH pulse frequency was decreased (Dong et al., 1991), and for diabetic humans, in which LH pulse frequency was also reduced (Djursing et al., 1985). The fact that pulsatile secretion of LH was at a higher frequency in diabetic cyclic gilts in the study reported here is even more paradoxical considering that testosterone was increased and negative feedback effects on LH could be expected, on the basis of studies with male diabetic rats (Chandrakeshar et al., 1991). In addition, loss of body mass comparable to that reported here, but achieved through nutrient restriction, has been shown to decrease LH pulsatility in gilts (Armstrong and Britt, 1987). Thus, the present results could be explained by a decreased sensitivity of the hypothalamus and pituitary of diabetic gilts to negative feedback by peripheral steroids.

It appears, from Expt 2, that sufficient oestradiol was not produced in diabetic gilts to trigger an LH surge. However, LH concentrations did increase during the period from day 18 to day 25, and the increases appeared to be coordinated, with at least 1 day between the increases. Again these studies contrast with those in rats, in which peripheral oestradiol was not affected by diabetes mellitus lasting for 8 days, but the LH response to GnRH was impaired (Kirchick et al., 1978, 1979).
The explanation for these increases is unknown and the sampling schedule did not permit complete characterization of LH patterns. However, the results are consistent with those of Expt 1, which are that acute diabetes, as used in our experimental model, does not impair LH as a prerequisite for decreased follicular development. Rather, the decreased follicular development appears to be the primary event. Studies that demonstrate detrimental effects of diabetes mellitus on GnRH involve longer durations (3 weeks or greater) than the present study, so they are not comparable to the relatively short duration of diabetes mellitus used here (Bestetti et al., 1985, 1989; Valdes et al., 1991).

We conclude that the reduced growth of follicles, abnormalities in follicular steroidogenesis, and failure to ovulate are consequences of the acute onset of diabetes during the period of preovulatory follicular growth and are evident 6 days after removal of insulin treatment. The lack of insulin was associated with lowered IGF-I concentrations in peripheral and intraovarian pathways, and the major consequences were increased incidence of macroscopic atresia and decreased oestradiol production. The acute effects of insulin withdrawal evidenced in the follicle appear to be direct because they were not preceded by negative effects on the hypothalamic-pituitary axis. We therefore conclude that atretic processes increase in ovarian follicles in response to the removal of insulin and that these local changes are probably independent of circulating gonadotrophins.

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