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Role of Ovarian Hormones in the Long-term Control of Glucose Homeostasis

Effects on Insulin Secretion

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Summary. The role of ovarian hormones in the long-term control of B-cell function in the mouse has been examined. Ovariectomised adult female mice were treated with daily subcutaneous replacement doses of oestradiol (5 μg/kg), progesterone (1 mg/kg), both hormones combined, or vehicle only for 15 weeks. Ovariectomy caused a 40% increase in plasma glucose concentrations during glucose tolerance tests, a 26% decrease in the plasma insulin response to glucose (2 g/kg IP) and a 32% decrease in the plasma insulin response to arginine (2 g/kg IP) compared with control mice. When islets from ovariectomised mice were incubated for 30 minutes in media containing 28 mmol/l glucose or 2.8 mmol/l glucose with 5 mmol/l arginine, insulin release was reduced by 23% and 31% respectively. Total pancreatic and islet insulin content were each decreased by 36%, and the number of B-cells was decreased by 39% in the ovariectomised mice. These detrimental effects of ovariectomy were partially or totally prevented by the oestradiol and progesterone treatments. The results indicate that ovarian oestrogens and progestogens may play an important role in the long-term maintenance of B-cell competence in the female mouse.

Key words: Ovariectomy, oestradiol, progesterone, glucose tolerance, insulin release, pancreatic islets, mouse.

It has been shown that ovariectomy increases the incidence and severity of experimental diabetes in female animals [1]. These effects are opposed by natural oestrogens, which produce islet hypertrophy and hyperplasia, and increased B-cell granulation [1–4]. In non-diabetic female animals physiological amounts of oestrogens or progesterone administered for 2 to 3 weeks lower blood glucose concentrations and increase circulating insulin levels [5–7].

However, a possible physiological role of ovarian hormones in the long-term control of B-cell function in the female has not been investigated. We have therefore examined the effects of prolonged (15 weeks) ovarian endocrine deficiency and oestradiol-progesterone replacement regimens on B-cell function in ovariectomised female mice.

Materials and Methods

Animals

Adult female albino mice (A. Tuck & Sons Ltd., Battlesbridge) were housed in an air-conditioned room at 22 ± 2 °C with a regular lighting schedule of 9.5 h light (0800–1730 h) and 14.5 h dark. The mice were supplied a standard pellet diet (Mouse breeding diet, Heygate & Sons Ltd., Northampton) and tap water ad libitum except prior to and during certain experiments, as indicated.

Chemicals

Oestradiol-17β and progesterone were purchased from Sigma Chemical Co., Poole, England. Insulin antiserum (double antibody batch K5402) was purchased from Wellcome Reagents, Beckenham, England; ^{125}I-insulin from the Radiochemical Centre, Amersham, England; mouse insulin (batch M20169) from Novo, Bagsvaerd, Denmark; and insulin-free bovine serum albumin (fraction V) from Armour Pharmaceutical Co., Eastbourne, England. Other chemicals were purchased from BDH Chemicals, Poole, England, and May and Baker, Dagenham, England.

Experimental Design

Mice were bilaterally ovariectomised under sodium pentobarbitone anaesthesia (45 mg/kg IP) at 10 weeks of age and treatments were begun 1 week later. Groups of mice were treated for

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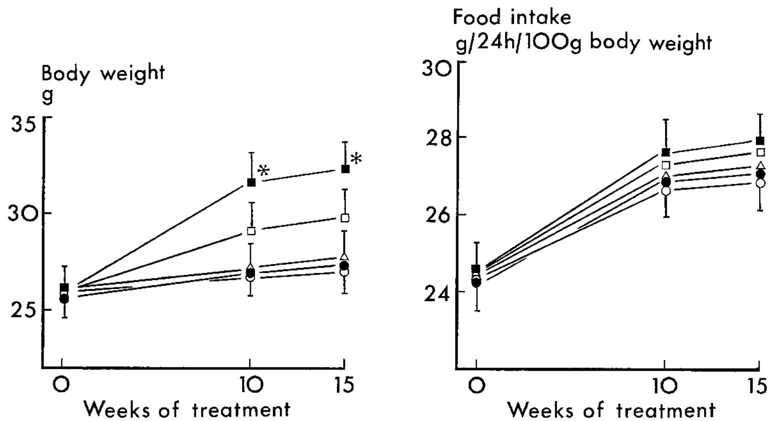


Fig. 1. Body weight and food intake in intact control (●), ovariectomised (■), oestradiol (○), progesterone (□) and oestradiol-progesterone (△) treated mice. Values are mean ± SEM of 12–15 mice. * $p < 0.05$ compared with control, oestradiol and oestradiol-progesterone groups

15 weeks with daily SC injections at 0900 h of either oestradiol-17β 5 μg/kg, progesterone 1 mg/kg, both hormones combined doses as previously, or vehicle only (0.8 ml/kg arachis oil). Intact control mice were sham-operated and treated with vehicle only. All tests were begun 24 h after the last hormone injection of week 15 of the treatment.

Since glucose homeostasis varies during the oestrous cycle, all tests on intact female mice were performed on the day of dioestrus, as determined by a vaginal smear [6]. Dioestrus corresponds to the lowest circulating levels of gonadal steroids [8].

Food Intake

Food intake was measured over 4 consecutive periods of 24 h during the week before ovariectomy, and during weeks 10 and 15 of treatment as described elsewhere [9].

Glucose and Arginine Tests In Vivo

Glucose tolerance tests were conducted on 12 h fasted mice and arginine tests on 24 h fasted mice. Food was withheld during the tests. Glucose (2 g/kg) or arginine hydrochloride (2 g/kg) was given IP. Blood samples (100 μl) were obtained from the cut tip of the tail immediately before the test substance was injected, and at 30 and 60 min after glucose, and at 15 and 30 min after arginine. Plasma was separated and analysed for glucose and insulin.

Incubation and Perifusion of Isolated Islets

Mice were fasted for 24 h and killed by decapitation. The splenic portion of the pancreas was removed and islets of similar size were isolated by microdissection. Gey and Gey buffer [10], pH 7.4 containing bovine serum albumin (0.2 mg/ml), glucose and arginine as described below, was used throughout.

For incubation experiments groups of 3 islets were transferred to vials containing 2 ml of medium containing 2.8 mmol/l glucose. The medium was preoxygenated with a gas phase of O_2 and CO_2 (95:5). Preincubations were performed at 37 °C for 30 min with constant gentle agitation and oxygenation. The medium was then removed and the islets were washed twice with fresh medium. Test incubations were performed for 30 min under the same conditions as the preincubations, with either low glucose (2.8 mmol/l), high glucose (28 mmol/l) or low glucose and arginine (5 mmol/l). To allow for residual insulin from the preincubation, the test medium was sampled for insulin content at the beginning and end of the test incubation. The difference was expressed as insulin release in ng/ml/3 islets/30 min.

For perifusion experiments groups of 10 microdissected islets were transferred to a Millipore Filtration Unit, 1 ml capacity. The islets were preperifused at 500 μl/min for 30 min with oxygenated medium, 37 °C containing 2.8 mmol/l glucose. Test perifusions were performed for 30 min with medium containing 28 mmol/l glucose, and effluent medium was assayed for insulin at 0, 1, 2, 3, 4, 5, 10, 15 and 30 min. At the end of the perifusion, islets were dried at 105 °C for 24 h and weighed. Insulin release was expressed in ng/ml/μg islet tissue.

Insulin Content of Pancreas and Islets

Mice were fasted for 24 h and killed by decapitation. Insulin was extracted from either total pancreas or groups of 10 microdissected islets by the acid-ethanol procedure of Malaisse et al. [11]. Insulin content was expressed as μg/g or μg/mg wet weight of pancreas or islet tissue respectively.

Glucose and Insulin Assays

Samples of plasma, incubation and perifusion media, and tissue extracts were stored at −20 °C. Plasma glucose was determined by an automated glucose oxidase procedure (Beckman Glucose Analyzer, Beckman Riic Limited, High Wycombe) and insulin was determined by double antibody radioimmunoassay [12] using mouse insulin as standard. The sensitivity of the assay, as defined by Midgley et al. [13] was 0.18 ng/ml and the intra-assay coefficient of variation was 3.58%.

Islet Morphometry

Pancrea were fixed in Bouin's solution, dehydrated in ethanol and embedded in paraffin wax. Sections of 6 μm thickness were stained with aldehyde fuchsin [14] counterstained with 2% aqueous light green. Five sections from each of 6–9 pancrea per group were selected from the head through to the tail of the pancreas and assessed for islet number, islet size and B-cell number. Islets were counted using a squared graticule at × 250 magnification. The number of islets was expressed per cm^2 of pancreas section. To assess islet size the longest and shortest diameters were measured using a scaled graticule at × 250 magnification, and the average diameter was calculated. B-cells were counted using a squared graticule at × 400 magnification. The number of B-cells was expressed per mm^2 of pancreas section.

Statistical Method

Groups of data were compared using Student's t test. Differences were considered to be significant if $p < 0.05$.

Results

Body Weight and Food Intake (Fig. 1)

Body weight was elevated in ovariectomised mice compared with all other groups except those treated with progesterone alone. Treatment with progesterone alone did not lower body weight significantly. Food intake was similar in the five groups of mice at the time intervals studied (10 and 15 weeks).

Fed and Fasting Plasma Glucose and Insulin (Table 1)

Fasting plasma glucose concentrations were consistently higher in ovariectomised mice than in control mice. Treatment with the three hormone regimens lowered glucose concentrations compared with the ovariectomised group. In the fed state there were no significant differences between the five groups of mice.

Plasma insulin concentrations were not significantly altered in fed or fasting ovariectomised mice compared with control mice. Treatment with the three hormone regimens significantly raised insulin concentrations in the 6- and 12-hour fasted states compared with both control and ovariectomised mice.

Glucose Tolerance (Table 2)

Glucose tolerance was impaired in ovariectomised mice, whereas tolerance in the three groups of hormone treated mice was similar to controls. The plasma insulin response to glucose was diminished in the ovariectomised mice, while mice treated with oestradiol alone and the combined oestradiol-progesterone regimen showed a greater response than control mice.

Arginine Administration In Vivo (Fig. 2)

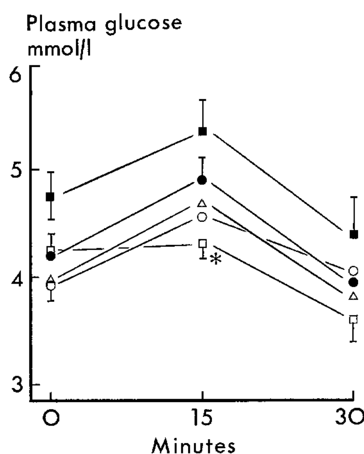
Arginine administration slightly raised and then lowered plasma glucose concentrations in all mice. Ovariectomy did not significantly increase glucose concentrations, although progesterone lowered glucose concentrations. The plasma insulin response to arginine was reduced in ovarectomised mice and restored in the three groups of hormone treated mice.

Table 1. Plasma glucose and insulin concentrations in fed, 6h, 12h, 18h and 24h fasted intact control, ovariectomised, oestradiol, progesterone and oestradiol-progesterone treated mice. Values are mean ± SEM of 12–15 mice. $^a p < 0.05$ compared with control group; $^b p < 0.05$ compared with ovariectomised group; $^c p < 0.05$ compared with control and ovariectomised groups

Animal preparation	Plasma glucose mmol/l					Plasma insulin ng/ml				
	Fed	6 h fasted	12 h fasted	18 h fasted	24 h fasted	Fed	6 h fasted	12 h fasted	18 h fasted	24 h fasted
Control	6.83±0.44	6.22±0.25	5.72±0.26	5.00±0.24	4.16±0.22	1.92±0.21	1.31±0.17	1.03±0.14	0.84±0.12	0.61±0.09
Ovariectomised	7.61±0.46	7.11±0.34^a	6.33±0.22^a	5.78±0.23^a	4.79±0.26^a	2.01±0.28	1.35±0.15	1.14±0.11	0.95±0.10	0.75±0.13
Oestradiol	6.94±0.45	6.27±0.26^b	5.61±0.23^b	5.05±0.23^b	3.83±0.25^b	2.35±0.32	1.84±0.16^c	1.42±0.10^c	0.96±0.13	0.82±0.14
Progesterone	7.00±0.47	6.32±0.25	5.77±0.21^b	5.11±0.22^b	4.22±0.23^b	2.24±0.33	1.66±0.11^c	1.45±0.12^c	1.04±0.11	0.85±0.15
Oestradiol-progesterone	6.77±0.38	6.26±0.24^b	5.75±0.21^b	4.94±0.27^b	3.89±0.25^b	2.12±0.27	1.72±0.13^c	1.44±0.12^c	1.02±0.12	0.75±0.09

Table 2. Plasma glucose and insulin concentrations during intraperitoneal glucose tolerance tests in 12 hour fasted intact control, ovariectomised, oestradiol, progesterone and oestradiol-progesterone treated mice. Values are mean ± SEM of 8–9 mice. $^{a}p < 0.02$, $^{b}p < 0.05$ compared with all other group; $^{c}p < 0.05$ compared with control and ovariectomised groups; $^{d}p < 0.05$ compared with ovariectomised group

Animal preparation	Plasma glucose mmol/l			Plasma insulin ng/ml		
	0 min	30 min	60 min	0 min	30 min	60 min
Control	5.68±0.27	9.94±0.61	6.38±0.45	1.01±0.13	3.27±0.21	1.85±0.19
Ovariectomised	6.37±0.23^{b}	12.76±0.72^{a}	8.61±0.56^{b}	1.15±0.10	2.53±0.26^{b}	1.64±0.19
Oestradiol	5.65±0.26	9.27±0.50	6.66±0.49	1.44±0.12^{c}	3.93±0.15^{c}	2.18±0.13^{d}
Progesterone	5.77±0.24	9.45±0.55	6.88±0.44	1.48±0.13^{c}	3.71±0.25	1.98±0.24
Oestradiol-progesterone	5.70±0.21	8.94±0.62	6.50±0.43	1.47±0.13^{c}	4.01±0.22^{c}	2.43±0.18^{c}



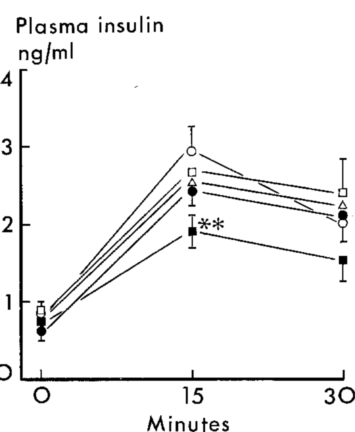


Fig. 2. Plasma glucose and insulin concentrations during intraperitoneal arginine administration tests in 24-hour fasted intact control (●), ovariectomised (■), oestradiol (○), progesterone (□) and oestradiol-progesterone (△) treated mice. Values are mean ± SEM of 9 mice. * $p < 0.05$ compared with ovariectomised group: ** $p < 0.05$ compared with all other groups

Table 3. Insulin release during incubation of islets isolated from 24 hour fasted intact control, ovariectomised, oestradiol, progesterone and oestradiol-progesterone treated mice. Values are mean ± SEM of 9 determinations. $^{a}p < 0.05$ compared with all other groups; $^{b}p < 0.05$ compared with control and ovariectomised groups

Animal preparation	Insulin release ng/ml/3 islets/30 min		
	Glucose 2.8 mmol/l	Glucose 28 mmol/l	Glucose 2.8 mmol/l and arginine 5 mmol/l
Control	2.24±0.26	7.73±0.49	10.81±0.73
Ovariectomised	2.01±0.47	5.92±0.59^{a}	7.40±0.85^{a}
Oestradiol	2.42±0.39	10.23±0.94^{b}	10.97±1.10
Progesterone	2.52±0.41	9.28±0.96	11.20±0.93
Oestradiol-progesterone	2.49±0.36	10.70±0.86^{b}	11.41±1.14

Insulin Release In Vitro

Incubations (Table 3). Basal insulin release from islets incubated for 30 minutes in the presence of 2.8 mmol/l glucose was not significantly altered by ovariectomy, or by the three hormone treatments. Glucose-stimulated insulin release (28 mmol/l glucose) was reduced by ovariectomy and restored by each of the hormone treatments. Similarly, the insulin response to arginine (5 mmol/l) in the presence of 2.8 mmol/l glucose was reduced by ovariectomy and restored by the hormone treatments.

Perifusion (Fig. 3). A square-wave increase in the glucose concentration of the perifusion medium from 2.8 to 28 mmol/l produced a biphasic release of insulin from islets of all five groups of mice. Both the first (1–5 min) and the second (5 min onwards) phases of

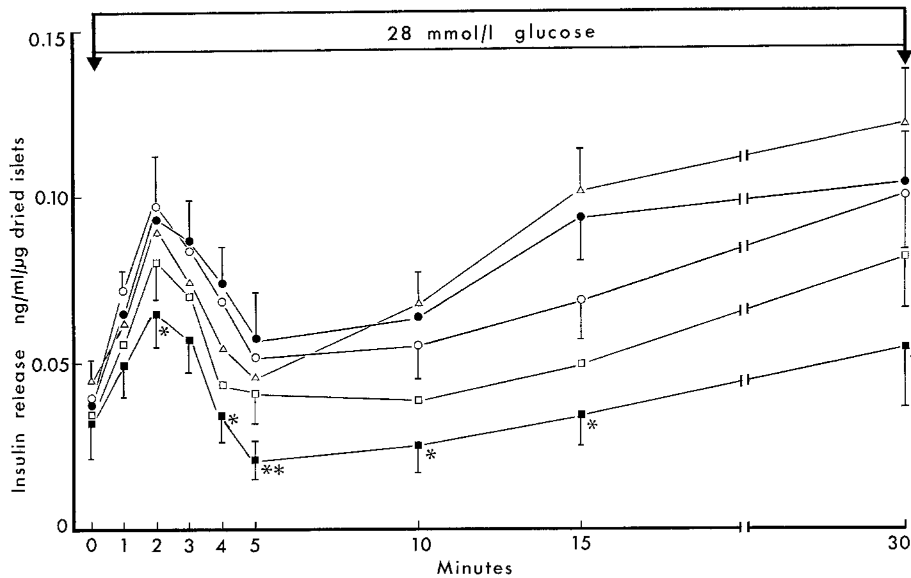


Fig. 3. Insulin release during perifusion of islets isolated from 24 hour fasted intact control (●), ovariectomised (■), oestradiol (○), progesterone (□) and oestradiol-progesterone (△) treated mice. The mice were treated for 15 weeks. Details of the perifusion procedure are given in the Materials and Methods section of the text. Values are mean ± SEM of 9 determinations. * $p < 0.05$ compared with control, oestradiol and oestradiol-progesterone groups; ** $p < 0.05$ compared with all other groups

Table 4. Insulin content of total pancreas and isolated islets, islet number, islet size and B-cell number in intact control, ovariectomised, oestradiol, progesterone and oestradiol-progesterone treated mice. Insulin content of total pancreas and isolated islets was determined after a 24 hour fast: values are mean ± SEM of 5–6 mice. Values for islet number, islet size and B-cell number are mean ± SEM of 5 pancreas sections from each of 6–9 mice. $^{a}p < 0.02$, $^{b}p < 0.05$ compared with all other groups, $^{c}p < 0.05$ compared with control and progesterone groups, $^{d}p < 0.05$ compared with ovariectomised group

Animal preparation	Total pancreatic insulin content µg/g wet wt	Islet insulin content µg/mg wet wt	Islet number/cm^2 pancreas secretion	Islet size average diameter µ	B-cell number/mm^2 pancreas section
Control	139±21	21.7±1.2	45±9	103±11	148±13
Ovariectomised	88±12^b	13.8±1.8^a	36±5	90±10	89±11^b
Oestradiol	197±42	25.2±1.3^c	53±6^d	104±10	131±12
Progesterone	141±18	20.1±1.1	40±5	95± 9	118±12
Oestradiol-progesterone	180±33	22.6±1.6	46±5	101± 9	134±17

insulin release were diminished by ovariectomy and partially or totally restored by the three hormone regimens.

Insulin Content of Pancreas and Islets (Table 4)

Total pancreatic insulin and the insulin content of the islets were considerably reduced in ovariectomised mice. The hormone treatments increased the insulin content of both total pancreas and the islets. Treatment with oestradiol produced a greater effect than progesterone, and the combined oestradiol-progesterone treatment produced an intermediate effect.

Islet Number and Size, and B-cell Number (Table 4)

Islet number and size were not significantly different in ovariectomised and control mice. However, islet number was increased in oestradiol treated mice compared with ovariectomised mice. The number of B-cells was reduced by ovariectomy, while each hormone treatment opposed this effect.

Discussion

The results demonstrate that ovarian hormones play an important role in the long-term integrity of islet B-cells in the female mouse. In addition the data illustrate that replacement doses of ovarian hormones exert a beneficial long-term effect on glucose homeostasis, and influence the long-term control of body weight.

Studies in mice and other rodents have shown that ovariectomy temporarily increases food intake, whereas oestrogens (but not progesterone) cause a transient decrease in food intake [15–17]. Transient changes in food intake (during the first 2 to 3 weeks

of treatment) were not examined in the present study. However, food intake was not significantly different between the five groups of mice after 10 and 15 weeks. This indicates that any transient effects on food intake are restored by 10 weeks.

Ovariectomy produced a persistent increase in body weight which was totally prevented by replacement with oestradiol, but not progesterone. This is consistent with the short-term weight limiting effect of oestrogens described by others [15, 16]. Progesterone is reported to antagonise this effect of oestrogens, although progesterone does not appear to exert a significant effect on body weight (at physiological concentrations) in the absence of oestrogens [15, 17]. The lack of a significant effect of progesterone alone was confirmed in the present study, but the dose used was not sufficient to significantly antagonise the effect of oestradiol. It has been suggested that the weight-limiting effect of oestrogens is mediated by the hypothalamus and involves a readjustment to the set point for weight regulation [15, 18]. The present data indicate that this is achieved in the long-term without a measurable change in food intake.

Although transient fluctuations in food intake and small protracted adjustments of body weight are known to affect glucose homeostasis [19] the magnitude and duration of these changes were not sufficient to account for the marked alterations in glucose tolerance and B-cell integrity in the present study. Thus, the long-term effects of sex hormones on islet B-cells and gluco-regulation appear to be independent of food intake and body weight.

The deterioration of glucose tolerance and reduced plasma insulin response to glucose after long-term ovariectomy, and the improvements conferred by physiological replacement doses of oestradiol and progesterone are consistent with short-term studies in other rodents [5–7]. Since glucose-induced insulin release in vitro was also reduced by ovariectomy and restored by the hormone replacements, the changes in glucose tolerance may be attributed in part to changes in insulin secretion. However, it has been suggested that sex steroids influence insulin resistance. There are reports that oestrogens increase the sensitivity of adipose tissue and skeletal muscle to the actions of insulin [20], while progesterone marginally antagonises the hypoglycaemic action of insulin [21]. These effects remain to be clarified with long-term studies at physiological doses.

The effects of ovariectomy and sex hormone replacements on arginine-induced insulin release were similar to those on glucose-induced insulin release. It is notable that progesterone lowered the plasma glucose response to arginine, although other treatments did not produce a significant effect. This action of progesterone has been observed in other species and may reflect different effects of oestrogens and progesterone on the handling of amino acids by muscle and liver [22, 23].

Perifusion studies revealed that both the first and second phases of glucose-stimulated insulin release are reduced in the long-term absence of ovarian hormones, and partially or totally returned by replacement with physiological doses of oestradiol and progesterone. Larger doses administered for shorter periods do not consistently reproduce these effects [24, 25]. Addition of oestradiol and progesterone to the islets in vitro has shown that an immediate and direct effect on insulin secretion does not occur ([5, 26, 27,] Lenzen, S., personal communication, 1979), but the effects of these steroids are evident 6 hours after administration in vivo [26]. Progesterone, but not oestradiol, has been shown to increase insulin release directly after 6 hours [26] and after 20 hours [27]. Progesterone has also been shown to enter the nuclei of islet cells [28] and increase islet cyclic AMP levels [29], although it does not appear to alter adenylate cyclase activity [27].

In addition to a direct effect on the islet B cells, sex hormones may influence insulin secretion via their effects on other endocrine glands. Oestrogens have been shown to increase circulating concentrations of growth hormone and free cortisol [4, 30, 31] which would tend to increase plasma insulin concentrations [32]. Conversely there is evidence that progesterone depresses growth hormone secretion [22]. Since the protective action of oestrogens in experimentally diabetic rats occurs after hypophysectomy and adrenalectomy [1, 2] it appears that pituitary and adrenal hormones do not represent an important mode of action of oestrogens on glucose homeostasis.

The islet insulin content offers a guide to insulin biosynthesis and storage. Ovariectomy reduced islet insulin content whereas oestradiol and progesterone opposed this effect. This is consistent with previous evidence that oestrogens increase B-cell granulation [1–3]. The changes in islet insulin content may be correlated with changes in islet morphometry. Within the limits of the histological technique the present data show a considerable fall in B-cell number in ovarectomised mice. All three hormone treatments countered this effect, but the oestradiol-containing regimens were consistently more effective. The design of the present study does not allow us to distinguish whether sex hormones stimulate B-cell replacement, reduce B-cell loss, or promote islet neo-formation. However, studies in non-diabetic and experimentally diabetic rats have indicated that oestrogens possess the ability to increase B-cell division

and islet neo-formation [1–3]. This would account for the beneficial effects of oestrogens in experimental diabetes.

In conclusion, we have shown that normal ovarian endocrine activity is important for the long-term functional integrity of the islet B-cells in the female. The present study substantiates evidence that endogenous sex hormone secretions modulate B-cell activity and glucose homeostasis during the reproductive cycle [6] and pregnancy [5, 33, 34]. The study also draws attention to the view that conditions of prolonged ovarian endocrine deficiency, such as the climacterium, may provide a diabetogenic stress which could be relieved by an appropriate hormone replacement regime.

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