EXPERIMENTAL STUDY

Effect of estradiol on insulin secreting INS-1 cells overexpressing estrogen receptors

P A Horn, M Möhlig, M Osterhoff, S Wolter, J Hofmann¹, C Stocking¹, W Ostertag¹, M Wahl², H Schatz and A Pfeiffer

Department of Internal Medicine, Medizinische Klinik und Poliklinik, Klinikum Bergmannsheil, D-44789 Bochum, Germany, ¹Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistr. 52, D-20251 Hamburg, Germany and ²Pharmazeutisches Institut, Universität Tübingen, Auf der Morgenstelle 8, D-72076 Tübingen, Germany

(Correspondence should be addressed to A Pfeiffer, Department of Internal Medicine, Medizinische Klinik und Poliklinik, Klinikum Bergmannsheil, Bürkle-de-la-Camp Platz 1, D-44789 Bochum, Germany; Email: andreas.pfeiffer@ruhr-uni-bochum.de)

(P A Horn is now at Clinic I for Internal Medicine, University of Cologne, Joseph-Stelzmann-Str. 9, D-50924 Köln, Germany)

Abstract

Background: Estrogen has been shown to have profound effects on insulin and glucose metabolism *in vivo*. Indeed, estrogens were recently shown to modulate ion channel and secretory activities in endocrine cells.

Design and Methods: To investigate whether estrogenic influences are caused by direct effects on pancreatic β -cells, we equipped INS-1 insulinoma cells with estrogen receptors and monitored insulin content and Ca^{2+} fluxes as well as basal and stimulated insulin secretion upon different stimuli including glucose, the Ca^{2+} ionophore ionomycin, the Ca^{2+} channel agonist BayK8644, the protein kinase C activator TPA, and the adenylate cyclase activator forskolin.

Results and Conclusion: Our data reveal that estradiol has no significant direct effect on proliferation rate, insulin content, basal and stimulated insulin output as well as Ca^{2+} fluxes of insulin secreting cells *in vitro*, indicating that *in vivo* responses to estrogen on insulin and glucose metabolism result from indirect betacytotropic effects.

European Journal of Endocrinology 142 84–91

Introduction

Estrogen is known to have beneficial effects on a range of metabolic risk factors for coronary heart disease (CHD) and the decline in estrogen concentrations at the menopause would be expected to have adverse effects (1). Links between glucose and insulin metabolism and CHD have long been suggested by an increased incidence of CHD and other vascular diseases in diabetes (2), particularly in female patients (3).

Ovarian endocrine insufficiency after the menopause has also been proposed to provide a diabetogenic influence (4). A large study on pre- and postmenopausal women revealed an association of menopause with a marked reduction in the pancreatic insulin response to glucose (5). Recently, a decrease in fasting levels of glucose has been reported upon administration of oral estrogen replacement in postmenopausal women (6).

In animal studies the main steroids of the ovary, the estrogens and the progestins, have been shown to provide a protective influence to the susceptibility to experimental diabetes (7, 8). Furthermore, an increase in basal glycemia and impaired glucose tolerance have been observed in ovariectomized mice as well as rats

and steroid replacement experiments then indicated that a deficiency of estrogens is mainly responsible for the deterioration of glucose tolerance (9, 10).

Transdermal estradiol replacement therapy in estrogen-deficient postmenopausal women was shown to improve β -cell function *in vivo* and to augment insulin secretion in response to an acute glucose challenge (11, 12).

This effect was proposed to involve a tropic action of estradiol on pancreatic islets in combination with an increase in glucose transport in muscle and an inhibition of gluconeogenesis (12). Indeed, the islets of Langerhans have been demonstrated to express estrogen receptors (10, 13) and to show a tropic response to estradiol treatment *in vivo* (14). These effects may either be caused by the estrogen itself or they may represent indirect effects due to hormonal changes in response to estrogen treatment. Few data exist on direct effects of estradiol treatment on isolated β -cells in culture. Sorenson and colleagues reported a slightly elevated basal insulin secretion after incubation of rat islets with estradiol for 24 h (15), while Nielsen and colleagues did not find a significant effect of estradiol on basal insulin release (16). *In vitro* experiments by Howell *et al.* (17) on

Figure 1 Design of the retroviral expression vector for the estrogen receptor. SV40 E/P, simian virus 40 promoter/enhancer; hER, human estrogen receptor.

glucose-stimulated insulin secretion found a significant influence of estrogen only in combination with progesterone. Therefore, the detailed role of estrogen on insulin secretion has remained unknown.

In the present study, glucose responsive INS-1 insulinoma cells which have maintained many of the specifics of primary β -cells, were equipped with estrogen receptors by retroviral gene transfer to allow a study of estradiol action on β -cells. The cells were also equipped with a lacZ gene under the control of an estrogen-responsive internal promoter to control for the efficient expression of estrogen receptors and to control for their effect on gene transcription.

Materials and methods

Chemicals were from Merck, Darmstadt, Germany or from Boehringer Mannheim, Mannheim, Germany, and were of analytical or higher grade except when noted otherwise. Geneticin (G418), ionomycin, forskolin and tetradecanoyl-12,phorbol-13-acetate (TPA) were from Calbiochem, San Diego, CA, USA and Fura-2-acetoxymethylester (Fura-2-AM) was from Molecular Probes, Eugene, OR, USA.

Cell culture

INS-1 cells (18) were kindly provided by C Wolheim and M Asfari, Geneva, and were cultured in RPMI1640 (Gibco, Gaithersburg, MD, USA) containing additions as described. All cells transduced with estrogen receptors were kept in phenol red-free medium containing charcoal-stripped fetal calf serum (FCS) (19). Fetal calf serum was stripped by treatment with 1% dextran-coated charcoal at 56 °C for 30 min. Thereafter, charcoal was sedimented and the FCS was sterilized by filtration through 0.22 μ m filters.

Design of retroviral vectors

Estrogen receptor vector The retroviral construct used for expression of the human estrogen receptor was composed of a mouse embryonic stem cell virus (MESV)-derived vector in which the original long terminal repeat

(LTR) is replaced by a myeloproliferative sarcoma virus long terminal repeat (MPSV-LTR) to allow for a higher expression level of the hygromycin resistance gene (hyg^R). The human estrogen receptor expression is driven by an internal early simian virus 40 (SV40) enhancer/promoter (20). Thus, both genes are coexpressed independently without a need for splice signals. To avoid termination of viral RNA at internal polyA sequences, both transcripts use the 3' LTR polyA signal (Fig. 1).

Estrogen-responsive retroviral construct In order to test for gene expression in response to estrogen stimulation, a retroviral construct called p5NMere was made based on a MPSV-derived vector (20 and C Stocking, J Hofmann and W Ostertag, unpublished data). This vector consisted of a neomycin resistance gene driven by the 5' LTR followed by a β -galactosidase gene driven by a stimulating protein (Sp1 element) in combination with three consecutive estrogen responsive elements (EREs) (Fig. 2).

Production of retrovirus and retroviral infection

All retroviruses used were replication deficient and therefore could only be provided by packaging cells. Stable producer cells were obtained by first transfecting the ecotropic producer cell line GP+E-86 (21) via lipofection using DOTAP (Boehringer Mannheim) according to the instructions of the producer. After 24, 36 and 48 h the supernatants from these cells were used to infect the amphotropic producer cell line PA 317 (22). The cells were selected with 800 μ g/ml G418 or 200 μ g/ml hygromycin for at least 14 days. Supernatants from these stable producer cell lines were used to repeatedly infect INS-1 insulinoma cells, which were consecutively also selected with the appropriate antibiotic.

Transient infection via electroporation

Estrogen receptor expression was controlled by transient transfection with a reporter plasmid pGI3P (Promega, Madison, WI, USA) which contains a minimal SV40 promoter. Two copies of the vitellogenin estrogen

Figure 2 Design of the estrogen-responsive retroviral expression vector for β -galactosidase. neo^R , neomycin resistance.

responsive element were cloned in front of the minimal SV40 promoter rendering the luciferase estrogen responsive. This vector was transiently transfected by electroporation together with a pSV- β Gal vector (Promega) to control for transfection efficiency. Electroporation was performed at 1000 μ F and 270 V in an Invitrogen electroporator II. Luciferase was analyzed as described (23). β -Galactosidase was quantified using the Galacto-Light kit (Tropix, Bedford, MA, USA) according to the protocol of the manufacturer. Measurements were performed in triplicate.

Histochemical β -galactosidase staining

An X-gal stain (5-brom-4-chlor-3-indolyl-(D-galactopyranoside)) was performed to demonstrate β -galactosidase expression on a single cell basis (24). Cell culture medium was removed and cells were fixed with 0.05% glutaraldehyde for 10 min at room temperature. Cells were washed three times with PBS and incubated overnight with X-gal staining solution (20 mmol/l $K_3Fe(CN)_6$, 20 mmol/l $K_4Fe(CN)_6$, 2 mmol/l $MgCl_2$, 1 mg/ml X-gal) at 37°C. β -Galactosidase-positive cells develop a characteristic blue staining.

Measurement of intracellular calcium concentrations

Cells were seeded on glass coverslips and cultured for 24 h in either the presence or absence of estrogen. For preloading the cells with the fluorescence indicator, the culture medium was removed and the cells incubated in Krebs–Ringer–HEPES solution containing Fura-2-AM (2 μ mol/l) and glucose (3 mmol/l) for 45 min at 37°C. After washing the cells three times with the experimental buffer, they were transferred to a temperature-controlled microscopic stage and perfused at 2 ml/min with experimental buffer consisting of Krebs–Ringer–HEPES containing (mmol/l) NaCl (122), KCl (4.8), $CaCl_2$ (2.5), $MgCl_2$ (1.1), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (10) with a pH adjusted to 7.4 and gassed with ambient air. Single cell fluorescence was measured at 340/380 nm excitation and 510 nm emission using an amplified CCD camera-based imaging system (IonOptix, Milton, MA, USA) operated by the IonWizzard software (IonOptix), at a sampling rate of two images per second. Zones of interest were set to cover whole cells and the average fluorescence of these zones was monitored. All results are presented as fluorescence ratio of the signal obtained with an alternating excitation of 340 or 380 nm. The traces shown are representatives for the indicated number of cells out of 5–6 different incubations.

MTT assay

Cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT; Sigma, Deisenhofen, Germany) assay according to standard protocols (25).

Insulin radioimmunoassay

A guinea pig antibody against human insulin was obtained from Sigma and used at a dilution of 1:5000, rat insulin was used to construct the standard curve. Incubations were performed overnight at 4°C and free insulin was separated using dextran-coated charcoal (26). The intra-assay coefficient of variation at 4 ng/ml insulin was 8% and the interassay coefficient of variation was 10%.

For intracellular insulin concentrations, cells were homogenized in ethanol containing 5% acetic acid (300 μ l/well) and extracted overnight. The extract was centrifuged for 30 min at 15 000 g, lyophilized and resuspended in assay buffer.

Western blot

Homogenate of the cells was separated by SDS-gel electrophoresis following standard procedures before protein transfer to polyvinylidene fluoride (PVDF)-membrane (27). Anti-human estrogen receptor antibody (AB-1, Oncogene Science, Cambridge, MA, USA) was used in a 1:1000 dilution for detection. Visualization was performed by using the ECL-system (Amersham-Pharmacia, Little Chalfont, Bucks, UK).

Statistical analysis

Statistical analysis was performed using SPSS software. Comparisons between with and without estrogen were performed with a paired *t*-test and multiple analysis of variance. A value of *P* < 0.05 was considered significant.

Results

Retroviral gene transfer to INS-1 cells

Expression of the estrogen receptor in INS-1 cells was shown by an immune-blot using a monoclonal mouse antibody against human estrogen receptor (Fig. 3a). However there was no estrogen-dependent gene expression in INS-1 cells detectable using an estrogen-responsive reporter construct (Fig. 3b). This was interpreted as a consequence of the low amount of estrogen receptor. We therefore constructed INS-1 cell lines overexpressing human estrogen receptors by retroviral transfer. Hygromycin resistance gene encoded by the same retroviral vector was used for selection. The cells were subcloned and two clones with strong estrogen-dependent gene expression were identified by transient transfection with an estrogen responsive luciferase reporter vector (Fig. 3b). These were then tested for glucose responsiveness of insulin secretion. One of the highly estrogen-responsive clones, INS-E3, showed an approximately 10-fold increase in insulin secretion in the presence of 16.5 vs 2.5 mmol/l glucose and was chosen for further experiments.

Figure 3 (a) Western blot of estrogen receptor expression. M, marker; A, homogenate of INS-1 cells; B, homogenate of INS-E3 cells overexpressing estrogen receptors. (b) Estrogen-inducible gene expression demonstrated by transient transfection of β -galactosidase. Two of the estrogen receptor transduced clones called INS E3 and INS E7 show a marked response (10.1-fold and 7.8-fold) to 10 nmol/l estradiol (E2) compared with an untransduced control (INS).

INS-E3 cells were infected with retroviral supernatants containing a vector which coded for the LacZ gene under the control of an estrogen response element and additionally a neomycin resistance gene. The cells were selected with 800 μ g/ml and were subsequently called INS-E3-LacZ.

The response to estrogen stimulation was greatly augmented in these cells. However, the estrogen response varied enormously between cells. X-gal staining of these

cells revealed that approximately 50% of the transduced cells express high amounts of β -galactosidase upon stimulation with estradiol (Fig. 4).

When β -gal expression was quantified in whole cell lysates, the INS-E3-LacZ cells showed an up to 130-fold induction of the β -galactosidase gene upon treatment with 10 nmol/l estradiol for 24 h compared with treatment with the estrogen antagonist ICI 182,780. The dose-response curve for estrogen was bell shaped

Figure 4 Estrogen-inducible expression of a retrovirally transfected β -galactosidase gene. Left panel, treatment with vehicle; right panel, treatment with 10 nmol/l estradiol.

Figure 5 Estrogen-inducible β -galactosidase activity in INS-E3-LacZ cells. Displayed is the relative β -galactosidase activity over background (untransduced INS-1 cells) after stimulation with the estrogen antagonist ICI 182,780 (ICI) or different estradiol (E2) concentrations. Each bar represents the mean $\pm$ S.E.M. from 3 separate experiments performed in triplicate. DMSO, dimethyl sulfoxide used as vehicle.

with smaller responses at 1 or 100 nmol/l estradiol (Fig. 5).

Changes in intracellular calcium

INS-1 cells respond to depolarization induced by elevated glucose or sulfonyl ureas with oscillations of intracellular Ca^{2+} -levels which are mediated by voltage-dependent Ca^{2+} -channels.

In order to elucidate possible effects of estradiol on these oscillations $[\text{Ca}^{2+}]_i$ was determined by the use of fluorescence changes of the Ca^{2+} -indicator Fura-2. INS-E3-LacZ cells were either grown in the absence of estradiol or pretreated for 24 h with 10 nmol/l estradiol to induce estradiol-dependent gene expression. Depolarisation of the cells with 30 mmol/l KCl, 16.7 mmol/l glucose or 100 $\mu\text{mol/l}$ tolbutamide induced the typical oscillations in $[\text{Ca}^{2+}]_i$ similar to those observed in primary β -cells. The oscillatory pattern remained unchanged in the cells pretreated with 10 nmol/l estradiol for 24 h (Fig. 6). Estradiol pretreatment also did not affect the additive effects of 16.7 mmol/l glucose plus 100 $\mu\text{mol/l}$ tolbutamide on intracellular Ca^{2+} levels.

Effects of estrogen on cell proliferation and insulin content in INS-E3-LacZ cells

Cell proliferation was not altered by up to 72 h of treatment with 10 nmol/l estradiol as assessed by cell counting and colorimetric formazan blue assays with MTT after seeding of 50 000 cells/1.9 cm^2 . The concentrations of glucose were similar with or without 10 nmol/l estradiol. The cellular content of insulin was not affected by 24 h of treatment with 10 nmol/l estradiol and was 342 ± 57 ng/well of 1.9 cm^2 in the

absence and 328 ± 54 ng/well in the presence of estradiol ($n = 7$).

Effects of estrogen on basal and induced insulin secretion in INS-E3-LacZ cells

Basal insulin secretion in the INS-E3-LacZ cells was low and amounted to $1.5 \pm 0.3\%$ of the cellular insulin content over 90 min in the absence ($n = 7$) and $1.3 \pm 0.2\%$ in the presence of 10 nmol/l estradiol ($n = 7$). Glucose at 16.5 mmol/l increased insulin secretion to $15.7 \pm 1.1\%$ of insulin content in the absence and to $14.2 \pm 1.9\%$ in the presence of estradiol over 90 min (Fig. 7). This corresponds to a 10.2- and 10.9-fold increase in the presence and absence of estradiol respectively, as compared with basal secretion at 2.5 mmol/l glucose.

To augment intracellular calcium concentrations, cells were treated with either the Ca^{2+} ionophore, ionomycin or the Ca^{2+} channel agonist, BAYk8644. Ionomycin (3 $\mu\text{mol/l}$) had no significant stimulatory effect on insulin secretion in the absence and presence of estradiol, whereas BAYk8644 increased insulin secretion to $4.1 \pm 0.4\%$ in the absence and to $3.9 \pm 0.5\%$ in the presence of 10 nmol/l estradiol ($n = 4$).

To examine the influence of estrogen on cAMP-mediated insulin secretion cells were stimulated with forskolin. Estradiol did not have a significant effect on forskolin-induced insulin secretion (9.7 ± 4.1 in the absence vs $8.3 \pm 1.0\%$ in the presence of estradiol).

The protein kinase C (PKC)-activating phorbol ester TPA increased insulin secretion to $8.4 \pm 5.8\%$ in the absence and to $5.5 \pm 1.3\%$ in the presence of 10 nmol/l estradiol. None of these differences reached statistical significance.

Figure 6 Effect of estrogen pretreatment on $[Ca^{2+}]_i$ of single INS-E3-LacZ-cells. The cells were cultured for 24 h in the absence (control) or presence of 10 nmol/l estrogen. KCl (30 mmol/l), glucose (2.8 or 16.7 mmol/l) or tolbutamide (100 μ mol/l) were added during the intervals indicated by the bars.

Discussion

Estrogen is known to influence the function of islets of Langerhans *in vivo*. Since it is not clear whether this is mediated by a direct influence on β -cells, we studied its effect on an insulinoma cell line. INS-1 cells were first equipped with human estrogen receptors by means of retroviral infection in order to augment any estrogen receptor-mediated responses. When these cells were subsequently infected with an estrogen-inducible control vector expressing β -galactosidase, we found a very high (approximately 130-fold induction) but heterogeneous expression according to the X-gal staining. This heterogeneity in expression levels after

retroviral gene transfer has been described previously and might be explained by either changes in the number of transcriptionally active mRNA templates due to differences in the formation of RNA polymerase II transcription complexes (28) or time-dependent differences in the inhibition of expression by methylation of cytosine residues in the LTR (29). The percentage of cells (being 50%) responding to an estrogen stimulus is probably underestimated since low levels of β -galactosidase expression are not detected with X-gal staining (30). In each case, estrogen receptor-mediated transcriptional responses were extensively induced in these cells.

Estradiol administration to rats leads to islet

Figure 7 Effect of estradiol (E2) on insulin secretion in response to different stimuli. Cells were incubated with 2.5 mmol/l ($n=8$) or 16.5 mmol/l ($n=4$) glucose (GLC), 0.1 μ mol/l TPA ($n=4$), 1 μ mol/l forskolin (FOR, $n=4$), 3 μ mol/l ionomycin (IONO, $n=3$) or 1 μ mol/l BAYk8644 (BAY-K, $n=4$). Each bar represents the mean $\pm$ S.E.M. from separate experiments performed in triplicate.

hypertrophy (31), similar to changes observed in islets in late pregnancy (32). In our experiments estradiol treatment did not have any influence on cell proliferation for up to 72 h. In these experiments we also controlled for glucose concentrations in the media since glucose metabolism affects the MTT assay which reflects mitochondrial activity (33).

Neither insulin content nor basal insulin secretion were significantly altered in the presence or absence of estradiol. None of the stimuli applied (high glucose, TPA for PKC-, forskolin for cAMP-, and ionomycin and BAYk8644 for Ca^{2+} -induced insulin secretion) resulted in a different insulin response whether estrogen was present or not.

Prolactin was previously shown to alter the proliferation and secretory behavior of β -cells (34, 35). Therefore, it appears likely that prolactin rather than estradiol plays a primary role in pregnancy-induced alterations of β -cell function.

Remarkably, estrogens exert profound effects on the electrical activity of endocrine cells. GH3 pituitary cells as well as gonadotropin-releasing hormone and opioidergic neuronal cells respond to steroid treatment with changes in spontaneous regular firing and with altered responses to neurotransmitter stimulation (36). These effects appear to be mediated by transcriptional and nontranscriptional pathways. However, there was no evidence for such responses in INS-1 cells although this insulinoma represents a glucose responsive and highly differentiated β -cell line.

In summary, our data reveal that estradiol has no significant direct effect on proliferation rate, insulin

content, basal and stimulated insulin output as well as Ca^{2+} -fluxes even in estrogen receptor overexpressing insulin secreting INS-1 cells. These *in vitro* data support the thesis that responses to estrogens on insulin and glucose metabolism seen *in vivo* may result from indirect rather than direct betacytotropic effects.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft Pf 164/13–2 and the Sonderforschungsbereich 545 to CS and WO. AP received a Hermann-and Lilly-Schilling professorship.

References

- 1 Spencer CP, Godsland IF & Stevenson JC. Is there a menopausal metabolic syndrome? *Gynecological Endocrinology* 1997 **11** 341–355.
- 2 Garcia MJ, McNamara PM, Gordon T & Kannel WB. Morbidity and mortality in diabetics in the Framingham population. Sixteen year follow-up study. *Diabetes* 1974 **23** 105–111.
- 3 Barrett-Connor EL, Cohn BA, Wingard DL & Edelstein SL. Why is diabetes mellitus a stronger risk factor for fatal ischemic heart disease in women than in men? The Rancho Bernardo Study (published erratum appears in *JAMA* 1991 **265** 3249). *JAMA* 1991 **265** 627–631.
- 4 Lenzen S & Bailey CJ. Thyroid hormones, gonadal and adrenocortical steroids and the function of the islets of Langerhans. *Endocrine Reviews* 1984 **5** 411–434.
- 5 Walton C, Godsland IF, Proudler AJ, Wynn V & Stevenson JC. The effects of the menopause on insulin sensitivity, secretion and elimination in non-obese, healthy women. *European Journal of Clinical Investigation* 1993 **23** 466–473.
- 6 Espeland MA, Hogan PE, Fineberg SE, Howard G, Schrott H,

- Waclawiw MA & Bush TL. Effects of postmenopausal hormone therapy on glucose and insulin concentrations. *Diabetes Care* 1998 **21** 1589–1595.
- 7 Rossini AA, Williams RM, Appel MC & Like AA. Sex differences in the multiple-dose streptozotocin model of diabetes. *Endocrinology* 1978 **103** 1518–1520.
 - 8 Nelson WO & Overholser MD. The effect of oestrogenic hormone on experimental pancreatic diabetes in the monkey. *Endocrinology* 1936 **20** 473–478.
 - 9 Bailey CJ & Ahmed-Sorour H. Role of ovarian hormones in the long-term control of glucose homeostasis. Effects of insulin secretion. *Diabetologia* 1980 **19** 475–481.
 - 10 El-Seifi S, Green IC & Perrin D. Insulin release and steroid-hormone binding in isolated islets of Langerhans in the rat: effects of ovariectomy. *Journal of Endocrinology* 1981 **90** 59–67.
 - 11 Yki-Järvinen H. Role of insulin resistance in the pathogenesis of NIDDM. *Diabetologia* 1995 **38** 1378–1388.
 - 12 Cagnacci A, Soldani R, Carriero PL, Paoletti AM, Fioretti P & Melis GB. Effects of low doses of transdermal 17 beta-estradiol on carbohydrate metabolism in postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* 1992 **74** 1396–1400.
 - 13 Tesone M, Chazenbalk GD, Ballejos G & Charreau EH. Estrogen receptor in rat pancreatic islets. *Journal of Steroid Biochemistry and Molecular Biology* 1979 **11** 1309–1314.
 - 14 Faure A, Haouari M & Sutter BC. Insulin secretion and biosynthesis after oestradiol treatment. *Hormone and Metabolic Research* 1985 **17** 378.
 - 15 Sorenson RL, Brelje TC & Roth C. Effects of steroid and lactogenic hormones on islets of Langerhans: a new hypothesis for the role of pregnancy steroids in the adaptation of islets to pregnancy. *Endocrinology* 1993 **133** 2227–2234.
 - 16 Nielsen JH, Jorgensen R, Jensen D & Brunstedt K. Direct long-term effects of natural and synthetic sexual steroids on pancreatic islets in tissue culture. *Acta Endocrinologica* 1980 **94** 63A (Abstract).
 - 17 Howell SL, Tyhurst M & Green IC. Direct effects of progesterone on rat islets of Langerhans *in vivo* and in tissue culture. *Diabetologia* 1977 **13** 579–583.
 - 18 Asfari M, Janjic D, Meda P, Li G, Halban PA & Wollheim CB. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 1992 **130** 167–178.
 - 19 Eckert RL & Katzenellenbogen BS. Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells. *Cancer Research* 1982 **42** 139–144.
 - 20 Hofmann J. Entwicklung retroviraler Vektoren zur induzierbaren Genexpression. (Development of retroviral vectors for inducible gene expression). *PhD Dissertation*. Universität Hamburg, Germany, 1995.
 - 21 Markowitz D, Goff S & Bank A. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *Journal of Virology* 1988 **62** 1120–1124.
 - 22 Miller AD & Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Molecular and Cellular Biology* 1986 **6** 2895–2902.
 - 23 Brasier AR, Tate JE & Habener JE. Optimized use of firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques* 1989 **7** 1116–1122.
 - 24 Alam J & Cook JL. Reporter genes: application to the study of mammalian gene transcription. *Analytical Biochemistry* 1990 **188** 245–254.
 - 25 Hansen MB, Nielsen SE & Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods* 1989 **119** 203–210.
 - 26 Hutton JC, Bailyes EM, Rhodes OJ & Guest PC. Regulation of biosynthesis and processing of polypeptide hormones. In *Peptide Hormone Secretion: a Practical Approach*, pp 309–336. Eds JC Hutton & K Siddle. Oxford: Oxford University Press, 1990.
 - 27 Möhlig M, Wolter S, Mayer P, Lang J, Osterhoff M, Horn PA *et al.* Insulinoma cells contain an isoform of Ca²⁺/calmodulin dependent protein kinase II delta associated with insulin secretion vesicles. *Endocrinology* 1997 **138** 2577–2584.
 - 28 Ko MS, Nakauchi H & Takahashi N. The dose dependence of glucocorticoid-inducible gene expression results from changes in the number of transcriptionally active templates. *EMBO Journal* 1990 **9** 2835–2842.
 - 29 Hoebe RC, Migchielsen AA, van-der-Jagt RC, van-Ormondt H & van-der-Eb AJ. Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation and is dependent on its chromosomal position. *Journal of Virology* 1991 **65** 904–912.
 - 30 Baum C, Forster P, Hegewisch-Becker S & Harbers K. An optimized electroporation protocol applicable to a wide range of cell lines. *Biotechniques* 1994 **17** 1058–1062.
 - 31 Costrini NV & Kalkhoff RK. Relative effects of pregnancy, estradiol, and progesterone on plasma insulin and pancreatic islet insulin secretion. *Journal of Clinical Investigation* 1971 **50** 992–999.
 - 32 Green IC & Taylor KW. Effects of pregnancy in the rat on the size and insulin secretory response of the islets of Langerhans. *Journal of Endocrinology* 1972 **54** 317–325.
 - 33 Janjic D & Wollheim CB. Islet cell metabolism is reflected by the MTT (tetrazolium) colorimetric assay. *Diabetologia* 1992 **35** 482–485.
 - 34 Boscher AC, Crepaldi SC, Carneiro EM, Delattre E & Atwater I. Prolactin induces maturation of glucose sensing mechanisms in cultured neonatal rat islets. *Endocrinology* 1993 **133** 515–520.
 - 35 Sorenson RL & Brelje TC. Adaptation of islets of Langerhans to pregnancy: β -cell growth, enhanced insulin secretion and the role of lactogenic hormones. *Hormone and Metabolic Research* 1997 **29** 301–307.
 - 36 Zakon HH. The effects of steroid hormones on electrical activity of excitable cells. *Trends in Neurological Science* 1998 **21** 202–207.

Received 7 April 1999

Accepted 18 August 1999