Glucocorticoids suppress β-cell development and induce hepatic metaplasia in embryonic pancreas

Chia-Ning SHEN*, Jonathan R. SECKL†, Jonathan M. W. SLACK* and David TOSH*[1]

*Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, U.K., and †Endocrinology Unit, Department of Medical Sciences, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, U.K.

Elevated glucocorticoids are associated with low birth weight and fetal 'programming' of hypertension and glucose intolerance. In the present paper, we show that treatment of fetal rats with dexamethasone during the last week of gestation reduces the insulin content of their pancreatic β-cells. We reproduce this effect of dexamethasone *in vitro* using organ cultures of mouse embryonic pancreas, and show that it is associated with an elevation of expression of the transcription factor C/EBPβ (CCAAT/enhancer-binding protein β) and a reduction of the transcription factor Pdx-1 (pancreatic duodenal homeobox-1). Dexamethasone also induces the appearance of hepatocyte-like cells in organ cultures of pancreas, based on the expression of liver markers, albumin, α1-antitrypsin and transthyretin. Evidence that C/EBPβ is responsible for compromising the differentiation and later function of β-cells is obtained from its effects on the β-cell-like cell line RIN-5F. Transfection with a constitutive form of C/EBPβ suppresses insulin formation, whereas introduction of a dominant-negative inhibitor of C/EBPβ has no effect. We conclude that dexamethasone inhibits insulin expression in pancreatic β-cells via a mechanism involving down-regulation of Pdx-1 and induction of C/EBPβ. This mechanism may operate in combination with other changes during fetal programming, leading to type 2 diabetes in later life.

Key words: CCAAT/enhancer-binding protein β (C/EBPβ), fetal programming, glucocorticoids, hepatic transdifferentiation, insulin, pancreatic duodenal homeobox-1 (Pdx-1).

INTRODUCTION

The concept of fetal 'programming' has been advanced to explain the higher prevalence of cardio-metabolic disorders, glucose intolerance and type 2 diabetes during adult life in individuals who had a low birth weight [1,2]. The underlying mechanisms are not defined, but animal studies and preliminary human evidence suggest that adverse events in early life may influence the neuroendocrine development of the fetus through the action of glucocorticoids [3]. Normally, the fetus has much lower levels of physiological glucocorticoid than its mother [4], a gradient ensured by 11β-HSD2 (11β-hydroxysteroid dehydrogenase type 2), an enzyme which rapidly inactivates physiological glucocorticoids (cortisol, corticosterone) to inert 11-keto forms in the placenta and fetal tissues [5]. Supra-physiological glucocorticoid doses have been shown to retard fetal growth, and human intrauterine growth retardation is associated with elevated cortisol levels ([6,7] and references therein).

Our recent studies have shown that treatment of pregnant rats with the synthetic glucocorticoid Dex (dexamethasone), which is a poor substrate for 11β-HSD2 and readily crosses the placenta, or with carbenoxolone, which inhibits 11β-HSD2, reduces birth weight. Although the offspring regain the weight deficit by weaning, in adulthood they exhibit permanent hypertension, hyperglycaemia, and increased hypothalamic-pituitary-adrenal axis activity [8–11]. This animal model of glucocorticoid programming provides an opportunity to examine the association between fetal growth and subsequent disorders in adult life, including type 2 diabetes. Although type 2 diabetes is often considered simply as a condition of insulin resistance of peripheral tissues, it also frequently involves some pancreatic pathology [12]. If pancreatic β cells are unable to respond to increased demands for insulin, then diabetes will frequently result.

We have also shown recently that Dex can cause transdifferentiation of pancreatic exocrine cells to hepatocytes. Transdifferentiation, defined as the direct conversion of one differentiated cell type to another [13,14], was shown most comprehensively for the cell line AR42J-B13, but we have also observed the expression of albumin in organ cultures of embryonic pancreas exposed to Dex [15–17]. We then set out to determine whether other liver markers were also expressed in embryonic pancreas following Dex treatment, as this would provide good evidence for transdifferentiation of normal fetal exocrine cells to hepatocytes, rather than simply the expression of a single liver protein in individual pancreatic cells. In the course of this work we found that, in addition to the induction of liver proteins, Dex also reduced the number of insulin-producing cells in the organ cultures.

There are two questions that arise. First, is there a permanent reduction of β-cell numbers, or a permanent reduction of β-cell function, or both? Secondly, what is the mechanism of the Dex effect? In the present paper, we show that Dex treatment of rats during fetal stage reduces the mass of β-cells, and also reduces the level of insulin production in the remaining cells in later life. We have gone on to investigate the mechanism of this effect in two *in vitro* systems. We have characterized more fully the effects of Dex on the mouse embryo pancreatic organ cultures and shown that there is hepatic differentiation and it is associated with upregulation of the transcription factor C/EBPβ

Abbreviations used: C/EBPβ, CCAAT/enhancer-binding protein β; DAPI, 4,6-diamidino-2-phenylindole; Dex, dexamethasone; DTT, dithiothreitol; GFP, green fluorescent protein; HBSS, Hanks balanced salt solution; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; LAP, liver activator protein; LIP, liver inhibitory protein; PBS-A, PBS (Dulbecco's solution A); Pdx-1, pancreatic duodenal homeobox-1; PFA, paraformaldehyde; RT, reverse transcriptase; TRITC, tetramethylrhodamine β-isothiocyanate.

[1] To whom correspondence should be addressed (e-mail D.Tosh@bath.ac.uk).

© 2003 Biochemical Society

(CCAAT/enhancer-binding protein β). We also show that there is a reduction of insulin-producing cells, also associated with elevation of C/EBPβ. We have gone on to show that introduction of C/EBPβ into the β-cell-like RIN-5F cell line will suppress insulin production.

The results therefore suggest that excess glucocorticoid is likely to elevate expression of C/EBPβ in the fetal pancreas and consequently lead to a reduction in β-cell efficacy.

MATERIALS AND METHODS

Cell lines and culture conditions

RIN-5F cell line, a subclone of the RIN-m rat islet cell line, was purchased from E.C.A.C.C. (European Collection of Animal Cell Cultures, Porton Down, U.K.). Cells were maintained in RPMI 1640 medium (Sigma, Poole, U.K.) containing 2 mM L-glutamine, 0.5 units/ml penicillin, 500 ng/ml streptomycin, and 10 % (v/v) fetal bovine serum. The medium was renewed every 2–3 days. Subculture was performed every 6–8 days at a ratio of 1:3–1:5. Cells were frozen and stored in fetal bovine serum containing 10 % (v/v) DMSO, and kept in liquid nitrogen or at − 80 °C. Dex was added as a solution in ethanol and the medium was changed every 2–3 days. RU486 was added at 2.5 μM, with the treatment commencing 1 h before addition of the Dex.

Embryonic culture of mouse pancreatic buds

Embryonic pancreas were isolated and cultured as described previously [18,19]. The dorsal buds were isolated from E11.5 mouse embryos. Briefly, coverslips coated with fibronectin were placed in 35 mm Petri plates containing BME (basal medium Eagle) medium with Earle's salts, 20 % fetal bovine serum and 50 μg/ml gentamycin (Life Technologies). A stainless steel ring of 3 mm internal diameter was placed over the fibronectin-coated area, and the pancreatic bud was dropped into the centre. To ensure spreading during culture, the buds were turned, if necessary, with a fine needle so that the cut surface lay down. Cultures were maintained for up to 7 days at 37 °C with 5 % CO_2, with a change of medium every day. The stainless steel ring was removed on the second day. Treatment of pancreatic cultures with Dex was performed from the third day of culture. This culture system enables the organ to grow as a flattened, branched structure suitable for whole-mount immunostaining.

Immunofluorescence analysis

For immunofluorescent staining, cells were cultured on non-coated glass coverslips, rinsed with PBS, fixed with 4 % PFA (paraformaldehyde) in PBS for 30 min. Cells were then permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 30 min, and incubated in 2 % blocking buffer (Roche), which contained 0.1 % Triton X-100, and then incubated sequentially with primary antibodies overnight at 4 °C and secondary antibodies for 3 h at room temperature. After incubation, cells were washed 3 times with PBS buffer, then mounted in Gelvatol medium [prepared by dissolving 20 g of polyvinyl alcohol in 80 ml of 10 mM Tris (pH 8.6), and 3 g of *n*-propyl gallate in 50 ml of glycerol followed by mixing and centrifugation at 7000 ***g*** to remove any undissolved particles]. Alternatively, for staining of pancreatic bud cultures, the pancreatic buds were fixed in MEMFA (10 % formaldehyde, 0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$) for 30–45 min at room temperature. They were washed three times with PBS and then stored in PBS at 4 °C for up to a few days. Prior to immunostaining, the cultures were treated with 1 % Triton X-100 in PBS and then blocked as above. The antibodies used were as follows (working dilutions in parentheses): rabbit polyclonal anti-amylase (1:300), guinea pig polyclonal anti-insulin (1:300), mouse monoclonal anti-glucagon (1:100), goat polyclonal anti-albumin (1:400), rabbit polyclonal anti-α1-antitrypsin (1:400), mouse monoclonal anti-goat/sheep IgG–FITC conjugate (1:150), and rabbit polyclonal anti-guinea pig IgG–TRITC (tetramethylrhodamine β-isothiocyanate) conjugate (1:300) all from Sigma Chemical Co. (St. Louis, MO, U.S.A.); rabbit polyclonal anti-transferrin (1:200), rabbit polyclonal anti-transthyretin (1:100) and swine polyclonal anti-rabbit IgG–TRITC conjugate (1:200) from DAKO (Ely, Cambridge, U.K.); rabbit polyclonal anti-GLUT2 (glucose transporter type 2) antibodies from Biogenesis (Poole, U.K.); mouse monoclonal anti-glutamine synthetase (1:100) from Becton Dickinson, mouse monoclonal anti-C/EBPβ (1:150) from Santa Cruz; mouse monoclonal anti-GFP (green fluorescent protein) (1:300) from Clontech; horse polyclonal anti-mouse IgG–FITC conjugate (1:150), horse polyclonal anti-mouse IgG–Texas Red conjugate (1:150), horse polyclonal anti-mouse IgG–AMCA (7-amino-4-methylcoumarin-3-acetic acid) conjugate (1:150), and goat polyclonal anti-rabbit IgG–FITC conjugate (1:150) from Vector Laboratories; rabbit polyclonal anti-mouse IPF (insulin promoter factor 1)/PDX antibody (1:100) was made by J. M. W. Slack against an 18-amino-acid C-terminal peptide conjugated to keyhole limpet haemocyanin. Specimens were observed either using a Zeiss confocal microscope (LSM510) or with a Nikon fluorescent microscope. Image collection from the Nikon was made with a Hamamatsu C4880 cooled CCD camera and the images were processed with Image Pro Plus and Photoshop.

For cell counting experiments, cells were fixed in 4 % PFA, immunostained for insulin, Pdx-1 (pancreatic duodenal homeobox-1) or C/EBPβ, and then stained with DAPI (4,6-diamidino-2-phenylindole). DAPI was dissolved in PBS at 500 μg/ml, and used at a dilution of 1:1000. Coverslips were incubated with DAPI (500 ng/ml) for 30 min at room temperature before being mounted on to slides in Gelvatol medium. Using the 40× objective of a Leica DMRB microscope, five fields were picked randomly from each dish and photographed with a digital Spot camera (Image Solutions). The cell number in each field was > 200. Statistical analyses was performed using the Student paired *t*-test in Excel. Significance was set at $P < 0.05$.

Live/dead cell assay

RIN-5F cells were seeded on to 35 mm dishes and grown for 5 days in culture medium supplemented with or without 1 μM Dex. The medium was removed and replaced with 2 ml of HBSS (Hanks balanced salt solution, without Phenol Red, Sigma), containing 320 nM Calcein AM and 1.6 μM ethidium homodimer (Molecular Probes), and then incubated at 37 °C for 30 min. After washing 3 times with PBS, 2 ml of HBSS was added to each dish, and images were then collected on a Zeiss confocal microscope (LSM510).

Insulin secretion assay

RIN-5F cells were plated in 24-well plates and grown for 5 days in culture medium supplemented with or without 1 μM Dex. The medium was removed, and cells were rinsed with 1 ml of Krebs–Ringer buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM Hepes, and 0.1 % bovine serum albumin, pH 7.4), and then

© 2003 Biochemical Society

incubated in 0.3 ml of Krebs–Ringer buffer containing 10 mM glucose for 2 h. At the end of the incubation period, the buffer was removed, centrifuged briefly, and the supernatant was used for measuring insulin secretion. For the measurement of total protein content, cells were washed twice with PBS, lysed with buffer containing 20 mM Hepes (pH 7.6), 150 mM NaCl, 1 mM EDTA, 2 mM DTT (dithiothreitol), 1 % Triton X-100, and 10 μg/ml proteinase inhibitors (leupeptin, aprotinin and pepstatin), and then the protein concentration of whole-cell extracts was determined using the Bio-Rad protein assay reagent. Measurement of insulin secretion was carried out with a rat insulin ELISA kit, according to the manufacturer's instructions (Mercodia, Uppsala, Sweden). Statistical analyses was performed on triplicate samples using the Student paired *t*-test in Excel. Significance was set at $P < 0.05$.

Transfection

Transient transfection with CMV-nucGFP (a plasmid containing GFP with a nuclear localization signal and under the control of the cytomegalovirus), LIP (liver inhibitory protein) and LAP (liver activator protein) used FuGENE 6 Transfection Reagent (Roche) with 2 μg of DNA per dish and incubation overnight at 37 °C 5 % CO_2. The medium was removed and the cells cultured for a further 72 h. Cells were fixed and immunostaining was performed as described above.

Preparation of whole-cell extracts, immunoprecipitation and Western blotting

Whole-cell extracts were prepared by lysing the cells with lysis buffer containing 20 mM Hepes (pH 7.6), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 1 % Triton X-100, and 10 μg/ml proteinase inhibitors (leupeptin, aprotinin and pepstatin). The whole-cell extracts were denatured, separated by 10 or 15 % polyacrylamide SDS/PAGE and transferred on to Hybond ECL nitrocellulose membrane (Amersham) and probed with rabbit polyclonal anti-mouse Pdx-1 (1:500, prepared by J. M. W. Slack) or mouse monoclonal anti-human-α-tubulin (1:1000, Sigma) antibodies. The signal was detected with an enhanced chemiluminescence kit (Amersham Biosciences).

For immunoprecipitation, 500 μg of whole-cell extracts was incubated with 5 μg of rabbit polyclonal anti-rat-C/EBPβ antibody (Santa Cruz Biotechnology) at 4 °C for 16 h. Protein A–agarose beads (Santa Cruz Biotechnology) were added to the samples and incubated for an additional 2 h at 4 °C. Agarose beads were collected by centrifugation at 12000 ***g*** for 1 min and washed three times with lysis buffer. Pellets were re-suspended in 15 μl of lysis buffer and 15 μl of 2× sample buffer, and then incubated for 10 min at 95 °C to release C/EBPβ protein. Samples were then separated by SDS/PAGE and immunoblotted with mouse monoclonal anti-human-C/EBPβ antibody (1:1000, Santa Cruz Biotechnology).

Histology and immunhistochemistry

Treatment of rats with glucocorticoid was performed as described previously [20]. *In vivo* studies were performed under the auspices of and in accordance with the U.K. Home Office's Animals (Scientific Proceedures) Act, 1986. Briefly, timed-mated pregnant Wistar rats were treated throughout the third week of gestation (from day 15 to day 21) with 100 μg/kg/day Dex or vehicle administered subcutaneously. Offspring were then killed 3 weeks postnatally. The pancreatic tissue was dissected free, fixed overnight in 4 % formaldehyde, and embedded in paraffin wax. Some representative sections (7 μm) were stained with haematoxylin and eosin (H&E). In addition, serial sections were prepared and immunohistochemically stained for insulin or glucagon. Briefly, sections were rehydrated and endogenous peroxidase activity blocked by incubating with 3 % H_2O_2 for 30 min. Sections were then blocked for 1 h in Roche blocking buffer prior to application of antibodies, and then incubated for 12–14 h at 4 °C with mouse monoclonal antibody raised against glucagon (Sigma), diluted at 1:300, or guinea-pig anti-bovine insulin antibody (Sigma), diluted at 1:200. Biotinylated anti-mouse or anti-guinea-pig IgG (Vector Laboratories) was diluted 1:300 in PBS-A [PBS (Dulbecco's solution A)] and applied for 1 h. The slides were then washed in PBS-A and incubated for 1 h with an ABC kit (Dako). Immunoreactivity was localized by incubation with DAB (3,3′-diaminobenzidine; 10 mg/15 ml of PBS), 0.01 % H_2O_2 for up to 5 min and the reaction stopped by washing in PBS. Tissue sections were counterstained with haematoxylin. Images of stained specimens were collected with a spot camera at 36 bit resolution and stored as 24-bit TIFF images.

RT (reverse transcription)-PCR

Total RNA was extracted using the TRI reagent (Sigma, Poole, U.K.). Pancreatic buds were cultured on fibronectin-coated coverslips without or with 1 μM Dex treatment for 5 days. The experiment was performed on two separate occasions with either 10 control or 10 Dex-treated buds in the first instance and 8 control or 8 Dex-treated buds in the second instance. Cells were lysed directly on to the culture dish by adding 0.05 ml of the TRI reagent. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogeneous lysate. After homogenization, the lysate from the pooled bud cultures was collected, and then 0.1 ml of chloroform was added to the tube. Covering the sample, it was shaken vigorously for 15 s and allowed to stand for 15 min at room temperature. It was then centrifuged at 12000 ***g*** for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube and 0.25 ml of isopropanol was added and mixed. The sample was allowed to stand for 5–10 min at room temperature, and then centrifuged at 12000 ***g*** for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed with 75 % ethanol. The RNA pellet was dried for 5–10 min, dissolved in water, and then stored at − 80 °C.

Before carrying out the cDNA synthesis, the RNA samples were digested with RQ-1 DNase (Promega, Southampton, U.K.) to remove any contaminating genomic DNA. First-strand complementary DNA was synthesized using SuperScript II RT (Invitrogen). The reactions were processed in a DNA thermal cycler under the following conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The number of cycles was 35 (32 cycles for C/EBPβ and α-actin). Primers were made by Invitrogen, and primer sequence were used as described previously [21–23]. PCR amplification was performed for insulin I (sense primer: 5′-GTCAGAAGGACTCC-TACGTG-3′; antisense primer: 5′-CCAGAGCTGTGATCTCCT-TC-3′), insulin II (sense primer: 5′ CCCTGCTGGCCCTGCTC-TT-3′; antisense primer: 5′-AGGTCTGAAGGTCACCTGCT-3′), Pdx-1 (sense primer: 5′-TGTAGGCAGTACGGGTCCTC-3′; antisense primer: 5′-CCACCCCAGTTTACAAGCTC-3′), glucokinase (sense primer: 5′-CCTGGGAACACATCTCCATG-3′; antisense primer 5′-CTTGCTTCTCTTCCCGGATC-3′), glucagon (sense primer: 5′-GCACATTCACCAGCGACTAC-3′; antisense primer: 5′-CTGGTGGCAAGATTGTCCAG-3′), somatostatin

© 2003 Biochemical Society

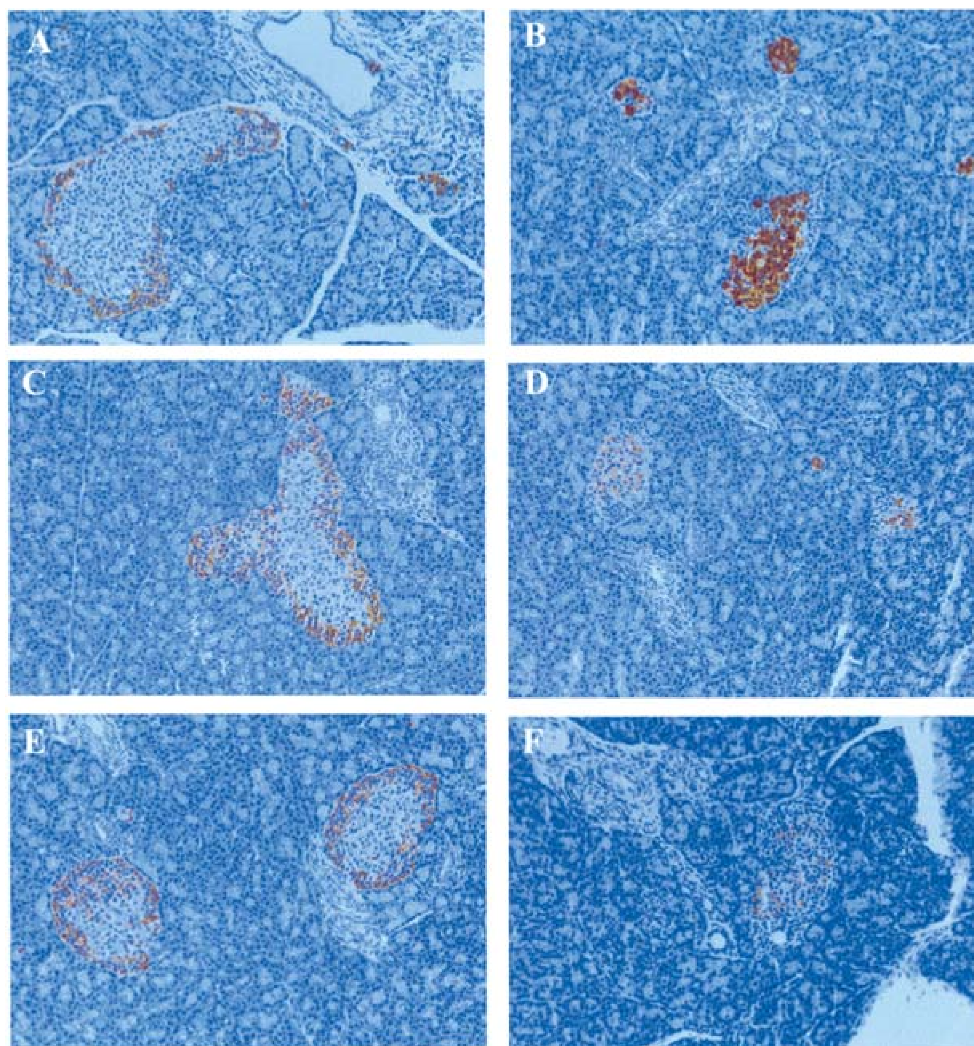

Figure 1 Glucagon or insulin immunostaining in sections of pancreas from control rats (A,B) or rats treated with Dex during the late gestation (C–F)

Typical fields of glucagon (**A**, **C**, **E**) and insulin (**B**, **D**, **F**) staining are shown.

(sense primer: 5′-CCGTCAGTTTCTGCAGAAGT-3′; antisense primer: 5′-CAGGGTCAAGTTGAGCATCG-3′), and amylase (sense primer: 5′-GTAGCAGGGTTCAGACTTGA-3′; antisense primer: 5′-TGCCACAAGTAGTGTCTGCA-3′), Pdx-1 (sense primer: 5′-TGTAGGCAGTACGGGTCCTC-3′; antisense primer: 5′-CCACCCCAGTTTACAAGCTC-3′), C/EBPβ (sense primer: 5′-ACAAGCTGAGCGACGAGTAC-3′, antisense primer: 5′-ACAGCTGCTCCACCTTCTTC-3′); α-actin (sense primer: 5′-GTCAGAAGGACTCCTACGTG-3′; antisense primer: 5′-CTTCCTCTAGTGTCGAGACC-3′).

RESULTS

Formation of abnormal islets *in vivo*

To study whether exposure to glucocorticoid during late gestation can cause alteration of pancreatic organization, we treated female pregnant rats with Dex throughout the third week of gestation (from day 15 to day 21), killed the offspring 3 weeks postnatally and then investigated the expression of insulin and glucagon by immunohistochemistry in control and Dex-treated offspring. Normally, glucagon-secreting α-cells are distributed around the periphery of the islet while the insulin-secreting β-cells represent the majority of the islet cells and fill its core (Figures 1A and 1B). The distribution and staining properties of α-cells was similar in control- and Dex-treated animals (Figures 1C and 1E) but the β-cells showed a markedly lower level of insulin expression compared with control (Figures 1D and 1F). The results suggest that β-cell activity is compromised in the offspring of Dex-treated rats and that this effect persists long after the end of the treatment.

Effects of Dex on pancreatic buds *in vitro*

The dorsal pancreatic buds were isolated from E11.5 mouse embryos and were allowed to attach on to fibronectin-coated coverslips overnight. The cultured pancreatic buds flattened out on to the substratum over the first 1–2 days and mesenchymal cells spread rapidly out of the explant to form a monolayer of cells surrounding the epithelia in the centre. On the second or third day, branches began to appear in the epithelium. Over the next 3 days, the epithelium became an extended branched structure radiating from the original centre, and development of exocrine cells could be recognized (Figures 2A and 2B). Insulin cells became more

© 2003 Biochemical Society

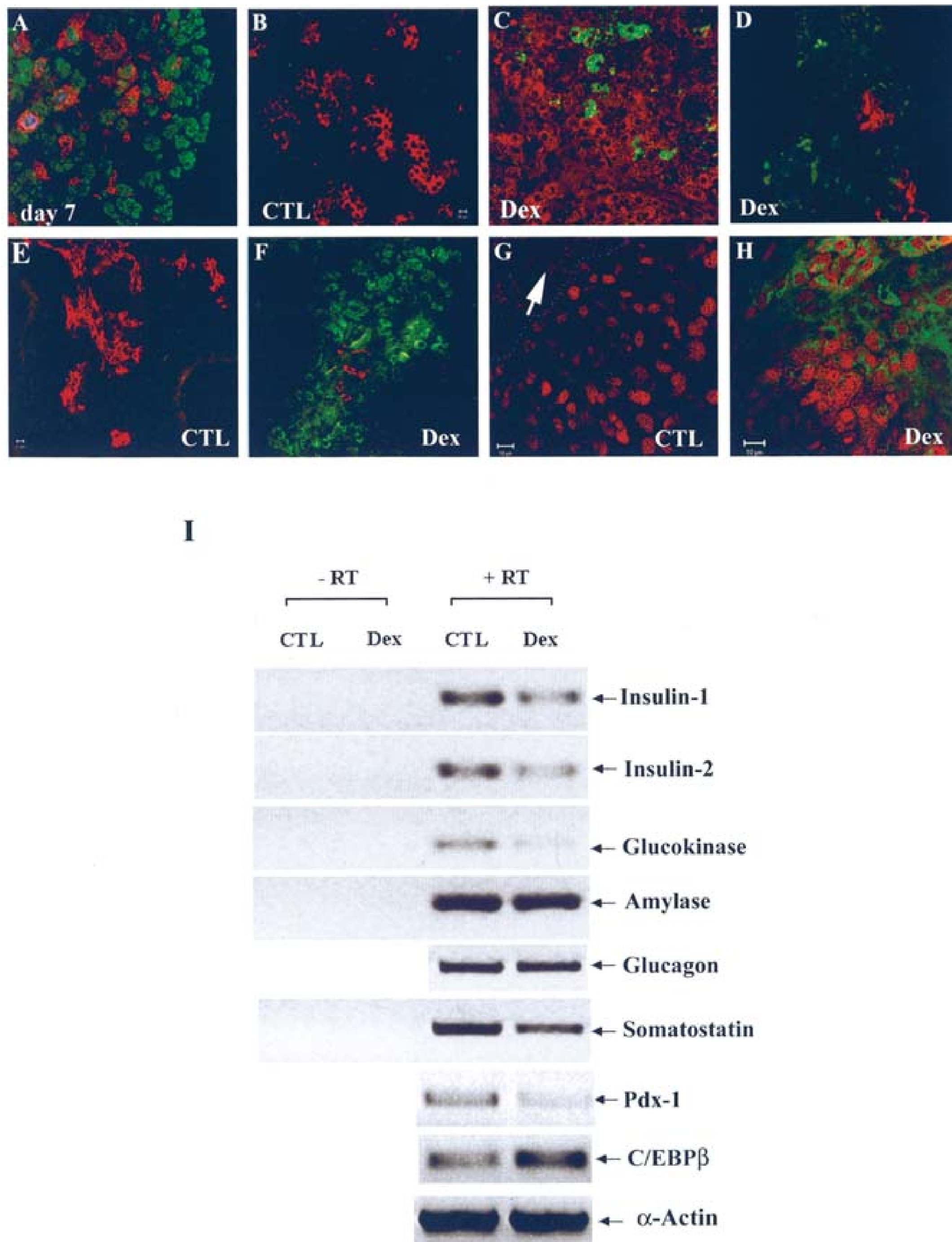

Figure 2 Development of pancreatic buds *ex vivo* and transdifferentiation of pancreatic cells to hepatocytes

(**A**) Buds were cultured for 7 days, fixed and stained for amylase (green), insulin (red) and glucagon (blue). To induce transdifferentiation, buds were cultured for 5 days in the absence (**B**, **E**, **G**) or presence (**C**, **D**, **F**, **H**) of 1 μM Dex, then immunostained for albumin (green)/amylase (red) (**B**, **C**), α_1-antitrypsin (green)/insulin (red) (**D**), transferrin (green)/insulin (red) (**E**, **F**), and albumin (green)/C/EBPβ (red) (**G**, **H**).Typical fields are shown. (**I**) Regulation of expression of pancreatic genes by Dex treatment. RNA was isolated from pooled mouse embryonic buds after treatment without or with Dex (5 days). RT-PCR was performed as described in the Materials and methods section. CTL, control.

numerous and strongly stained and some islet-like architecture can be seen from day 6 (Figure 2A). These results show that the cell differentiation in the cultures resembles quite closely what occurs *in vivo*, although it occurred slightly later in time, the delay being about 1 day over a 4-day culture period.

We showed previously that pancreatic tissue culture cells can be converted into hepatocytes by glucocorticoid treatment, and that pancreatic organ cultures would produce albumin under similar conditions [15]. However, it is not known whether the hepatocyte-like cells induced in the embryonic pancreas express other liver proteins. To test this idea in the present investigation, 2 days after isolation, 1 μM Dex was added to the embryonic pancreas cultures. This rapidly suppressed branching morphogenesis and, after treatment for 5 days, the appearance

© 2003 Biochemical Society

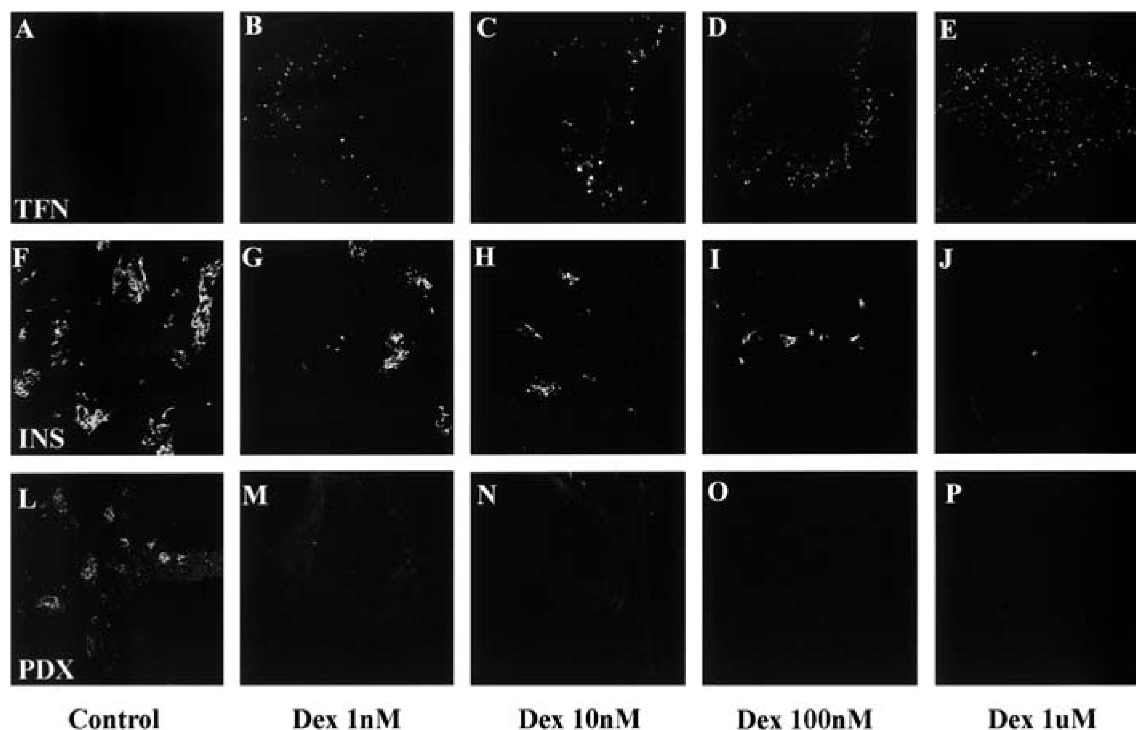

Figure 3 Effect of Dex (0–1000 nM) on liver and pancreatic gene expression in embryonic pancreatic buds

Buds were cultured for 5 days in the presence or absence of Dex, fixed and stained for transferrin (TFN; **A**–**E**), insulin (INS; **F**–**J**) or Pdx-1 (PDX; **L**–**P**). Typical fields are shown.

of hepatocytes could be seen, as judged from expression of albumin, α_1-antitrypsin and transferrin (Figures 2C, 2D and 2F respectively). At the same time it was also observed that the number of insulin-expressing cells was greatly reduced (Figures 2E and 2F). Because of its importance in pancreas-to-liver transdifferentiation, we examined expression of the transcription factor C/EBPβ. This is normally expressed in the mesenchyme but not the epithelium of the pancreatic cultures (Figure 2G). Following Dex treatment, it also became expressed in many epithelial cells and these tended to be co-expressed with albumin (Figure 2H).

We confirmed the results of the immunostaining by RT-PCR analyses (Figure 2I) of control and Dex-treated embryonic pancreatic cultures. After Dex treatment for 5 days, the expression of insulin I and II, and of glucokinase and *pdx-1* were reduced, whereas the expression of amylase, glucagon and somatostatin was not affected. The expression of C/EBPβ was increased following Dex treatment, consistent with the immunostaining results showing that expression was activated in epithelium, as well as mesenchyme. This shows that the Dex disproportionately affects the β cells compared with the exocrine or other endocrine cell types in the cultures.

Reduction of insulin-expressing cells

In order to investigate the dose-dependence of the glucocorticoid effect on pancreatic development, we added different concentrations of Dex (1 nM–1 μM) to the cultures and incubated for 5 days. Hepatocyte-like cells, as judged by transferrin staining, were found at all doses, but not in untreated controls (Figures 3A–3E).

To examine the effect on pancreatic β-cell development, we examined the expression of two pancreatic proteins: Pdx-1 and insulin. Pdx-1 is a key transcription factor in pancreatic development and is expressed in the whole pancreatic bud at early stages [21], but by 5 days of culture it is mainly expressed in insulin-producing cells and is known to be among the transcription factors regulating insulin expression in mature β-cells. Here, we found that Dex treatment caused a reduction of the proportion of cells expressing both Pdx-1 and insulin (Figures 3F–3P).

We next sought to find whether the changes in cell proportions required continuous exposure to Dex or whether they persisted in cultures that had been exposed to Dex for a limited period. To address this, we added 1 μM Dex to the buds at day 2, and cultured for 5 days as normal, then removed Dex and cultured for a further 4 days. At day 4 (2 days of Dex treatment), we found some cells expressing transferrin, accompanied by reduction of the formation of insulin-producing cells (Figures 4A, 4D and 4M). At day 7 (i.e. 5 days of Dex treatment), many cells expressed transferrin, and very few cells expressed insulin. By this stage, expression of Pdx-1 usually has been down-regulated in exocrine cells, and up-regulated in β-cells (Figure 4H) but treatment with Dex caused reduction of Pdx-1 expression in the whole bud (Figure 4K).

After removal of Dex from culture for 4 days, there was some recovery of insulin and Pdx-1 cells (Figures 4F and 4L), but we still found that some cells were expressing transferrin (Figure 4O), suggesting that the effect of the Dex treatment persists.

Suppression of insulin expression can occur via induction of C/EBPβ

In the present study, we showed that culture of embryonic pancreas with Dex induces C/EBPβ in many cells. To determine whether the induction of C/EBPβ is also involved in the down-regulation of insulin, we decided to simplify the system by using a pure culture of cells: the β-cell-like RIN-5F cell line. RIN-5F is a subclone derived from RIN-m cells, a widely used pancreatic β-cell-like line which expresses insulin. When the RIN-5F cells were treated

© 2003 Biochemical Society

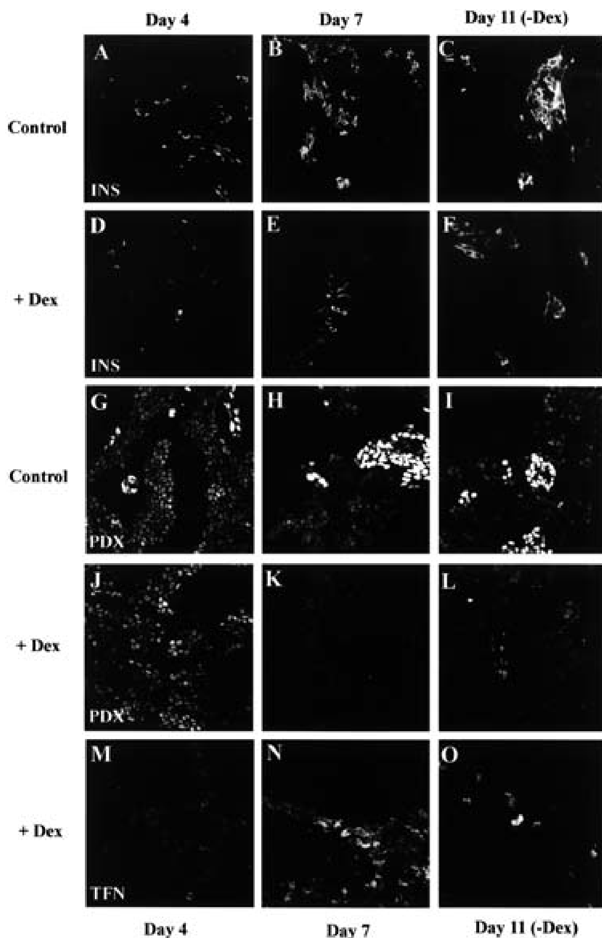

Figure 4 Time-course of insulin and Pdx-1 expression in embryonic pancreas

Buds were isolated from E11.5d mice embryos, and cultured for 2 days without Dex. Some buds were then treated with 1 μM Dex for 2 days (**D**, **J**, **M**), 5 days (**E**, **K**, **N**). For (**F**, **J** and **O**), buds were treated with 1 μM Dex for 5 days, after which the Dex was removed and the buds cultured in control medium for 4 days. Cultures were fixed at day 4, day 7 and day 11, and stained for insulin (INS; **A**–**F**), Pdx-1 (PDX; **G**–**L**) or transferrin (TFN; **M**–**O**). Typical fields are shown.

with 10 nM or 1 μM Dex for 4 days, C/EBPβ expression was induced and insulin expression was suppressed (Figures 5A–5C). Although there was some co-expression of C/EBPβ and insulin, most of the cells expressing C/EBPβ (red nuclei) lost insulin expression (green cytoplasm). When the cells were pretreated with the glucocorticoid receptor antagonist RU486, this completely blocked the expression of C/EBPβ and maintained the insulin expression (Figure 5D). These results suggest that the induction of C/EBPβ by Dex may play a role in suppression of insulin synthesis. The expression of Pdx-1 is also inhibited by Dex, although not to the same extent as insulin, as there are many cells co-expressing both Pdx-1 and C/EBPβ (Figures 5E–5G).

In order to quantify these results, we determined the effects of Dex on the number of RIN cells that expressed insulin, Pdx-1 or C/EBPβ in the absence and presence of Dex (Figures 6A–6F and 6I). There was a significant reduction in the proportion of cells expressing insulin and Pdx-1, and a significant increase in the proportion of cells expressing C/EBPβ following treatment with Dex ($P < 0.01$) (Figure 6I). In Western blot analyses, Dex induced both C/EBPβ and LAP, the truncated and constitutively active form of the factor. The expression of Pdx-1 decreased (Figure 6J).

We have considered whether these results might be artifacts arising from differential growth or death of subpopulations of cells in the cultures. Dex treatment does often inhibit cell growth, and for our RIN cultures, we found that over a 7-day culture period in Dex, the overall cell number was depressed to 45 % relative to controls. If a small minority of C/EBPβ^+ cells were to grow at the normal rate they might therefore double in relative frequency over this period. But the measured increase was actually 39-fold (Figure 6I), making this an unlikely possibility. In order to exclude possible effects of differential cell death, we used a live/dead cell assay to investigate whether any excess cell death accompanied the Dex treatment, but this showed no difference (Figures 6G and 6H). These various results all show that the Dex treatment causes a genuine reduction of expression of proteins associated with β-cell function. To find whether this resulted in functional impairment, we next examined the ability of glucose to stimulate insulin secretion in the RIN cells. Insulin was measured using a commercial test kit following exposure to 10 mM glucose, and it was found that the Dex-treated cells indeed showed a substantially lower response (Figure 6K).

To find the extent to which these Dex effects could be explained by the upregulation of C/EBPβ, we then examined the effects of introducing the constitutively active or dominant-negative forms of CEBP/β into the RIN cells. Figure 7(A) shows transfection of a plasmid expressing nuclear GFP from a CMV promoter. This is a control demonstrating that the transfection procedure itself does not affect insulin synthesis. Figure 7(B) shows the result of introducing LIP into RIN cells. LIP is a truncated form of C/EBPβ that acts as a dominant-negative inhibitor. This does not affect insulin synthesis. Figure 7(C) shows the introduction of LAP, another truncated form of C/EBPβ with constitutive activity. This suppresses insulin expression in those cells that express it. These experiments show that C/EBPβ inhibits insulin synthesis and make it likely that the glucocorticoids act, at least partly, by inducing C/EBPβ. We also attempted to rescue insulin production in Dex-treated cells by transfection with LIP. However, this was not successful (results not shown) and suggests that there are probably other parallel mechanisms besides the effects of C/EBPβ.

DISCUSSION

The present study had two aims: (i) to find whether excess glucocorticoid would affect pancreatic development *in vivo* and (ii) to further investigate the mechanism of the effect using *in vitro* models. The object of this work was to investigate a possible mechanism for fetal programming. It is possible that maternal stress, maternal Dex treatment or feto-placental 11β-HSD2 inhibition may lead to low birth weight and the programming of hyperglycaemia in adulthood [22]. Our study was carried out largely by immunohistochemical analyses because it has the advantage that it allows us to view the arrangement of exocrine and endocrine cells in the pancreas. But we have also made biochemical measurements by RT-PCR and Western blotting, to give an overall quantitative measure of the effects. The *in vivo* results show that administration of Dex during fetal development causes long-term damage to the β-cells, but not to α-cells or to the exocrine pancreas. Recent evidence provided by Breant and coworkers also complement our findings [23,24]. They show that undernutrition significantly increased maternal and fetal corticosterone levels. Fetuses (21-day-old) with undernutrition showed growth retardation and decreased pancreatic insulin content. What is more, adrenalectomy and subcutaneous corticosterone implants in their dams prevented the maternal

© 2003 Biochemical Society

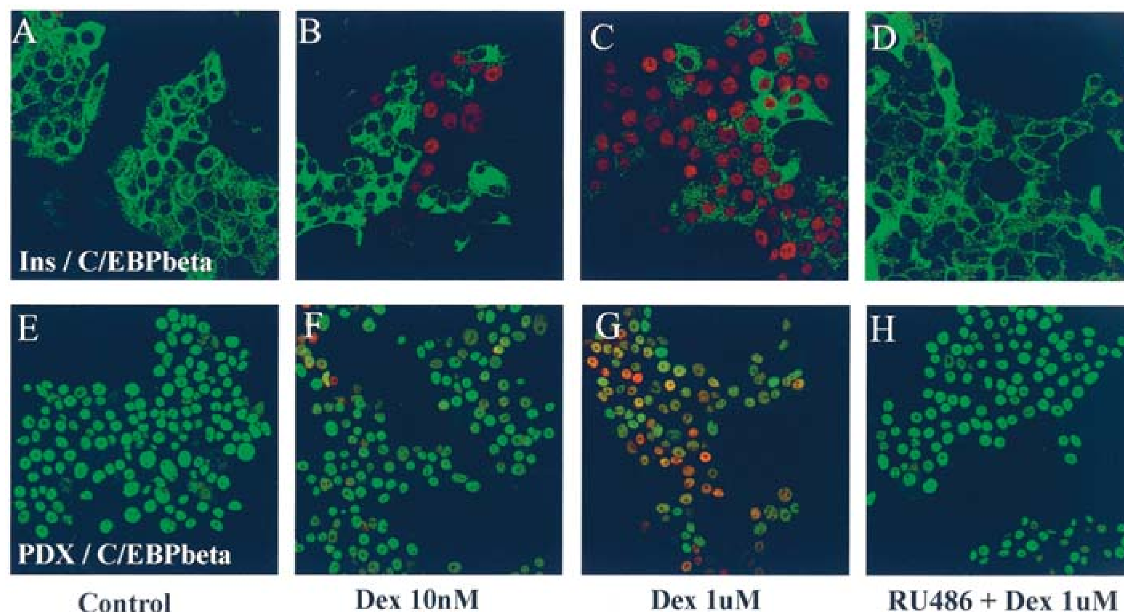

Figure 5 Dex treatment increases C/EBPβ and suppresses insulin expression in RIN-5F cells

Cells were incubated with 10 nM or 1 μM Dex for 4 days with or without pretreatment with 2.5 μM RU486. Cells were dual-stained with anti-insulin (green) and anti-C/EBPβ (red) antibodies (Ins/C/EBPbeta; **A**–**D**) or anti-Pdx-1 (green) and anti-C/EBPβ (red) antibodies (PDX/C/EBPbeta; **E**–**H**). Typical fields are shown.

corticosterone increase and restored fetal β-cell mass. Intriguingly, maternal undernutrition reduces placental 11β-HSD2, providing a commonality of mechanism for the two studies [25].

Our *in vitro* results also show that Dex compromises β-cell development. We have examined the effects on expression of Pdx-1 and of insulin in mouse embryo pancreatic organ cultures. Pdx-1 is a homeodomain protein that is expressed in the entire pancreatic anlagen at early stages. Jonsson et al. [26] showed that mice homozygous for a targeted mutation in the *pdx-1* gene selectively lack a pancreas. The mutant pups survive fetal development but die within a few days of birth. No pancreatic tissue or ectopic expression of insulin was detected in mutant embryos and neonates. In normal embryos, Pdx-1 expression declines during later embryonic stages in most of the pancreas but remains selectively expressed in the β-cells, where it binds to and transactivates the insulin promoter [27]. These findings show that Pdx-1 is needed for two distinct developmental events: first, the formation of the pancreas, and secondly for the differentiation of β-cells. The distinction is clearly shown in a study involving a late, β-cell-specific, targeted inactivation of the mouse *pdx-1* gene, which resulted in loss of the β-cell phenotype and consequently the development of maturity onset diabetes [28].

We show that the early, uniform, expression of Pdx-1 in pancreatic cultures is not affected by Dex. However the late, β-cell specific, phase is exquisitely sensitive, with most expression being suppressed by a dose of 1 nM. This is consistent with previous experiments using HIT cells which showed that glucocorticoid can repress Pdx-1 gene expression by interfering with HNF-3β activity on the islet-specific enhancer [29]. We show that the number of insulin-producing cells in the cultures is also greatly reduced by the Dex treatment, although a few cells form even at high doses (1 μM). There is a limit to the length of time that these cultures can be kept, but we have shown that the effect does persist for at least some days after withdrawal of the Dex, consistent with the long-term consequences seen *in vivo*. It should be noted that the peak concentration of Dex to which the fetal pancreas of our rats was subjected was in the region of 5 nM. This corresponds to the low end of the concentration range used for the organ culture experiments, but since we see clear *in vitro* effects at 1 nM, it is very likely that the same events occur *in vivo*.

Our results also provide a fuller documentation of the occurrence of hepatic transdifferentiation in embryonic pancreas exposed to glucocorticoid. Previous studies only showed the presence of albumin in some cells. In the present work, we find that this is accompanied by two other typical hepatocyte differentiation products: transferrin and α1-antitrypsin. This makes it highly likely that Dex does induce the formation of hepatocytes in these cultures. We also show the widespread induction of the transcription factor C/EBPβ, which is normally expressed in the liver but not in the pancreatic epithelium. We have previously shown that this factor alone can cause hepatic transdifferentiation in AR42J-B13 cells [15], and so it is probably an early step in the formation of hepatocytes in the organ cultures as well. Although Dex treatment can produce hepatic transdifferentiation, we have no current evidence that this occurred *in vivo*, probably because the dose experienced by the fetal rats was insufficient.

At the doses experienced *in vivo*, it is more likely that the main effect is some form of damage to the β-cells. Because of the importance of C/EBPβ in the induction of transdifferentiation, we suspected that it might also be responsible for the suppression of β-cell development. The organ cultures are not well suited to investigate this, as they consist of a mixture of cell types and are difficult to transfect with DNA. For this reason, we investigated this aspect further in the RIN-5F cells. In these cells, the transfection of LAP, the active form of C/EBPβ, is sufficient to suppress insulin expression, while the transfection of LIP, the dominant-negative form, has no effect. Previous studies have also shown that after exposure to high glucose, the β-cell lines HIT-T15 and INS-1 express high levels of C/EBPβ, which directly bind to rat insulin I promoter and inhibit the transcription of the gene [30]. Although this provides evidence of a role for C/EBPβ, we believe that other factors must also be involved because we have found that it is not possible to rescue insulin production in the Dex-treated RIN-5F cells by introduction of LIP. One possibility

© 2003 Biochemical Society

Figure 6 Dex suppresses insulin, Pdx-1 and increases C/EBPβ expression in RIN-5F cells

Cells were incubated without (**A**, **C**, **E**) or with (**B**, **D**, **F**) 1 μM Dex for 7 days. (**A**, **B**) Anti-insulin antibody (green) and DAPI (blue). (**C**, **D**) Anti-C/EBPβ antibody (red) and DAPI (blue). (**E**, **F**) Same fields as (**C**, **D**) Anti-Pdx-1 antibody (green) and C/EBPβ (red). (**G**, **H**) Live/dead cell assay. RIN-5F cells were seeded in 35-mm dishes and grown for 5 days in culture medium either without (**G**) or with (**H**) 1 μM Dex. Live cells positive for calcein are shown in green and apoptotic cells react with ethidium to give red. Typical fields are shown. (**I**) The graph shows cell counts of staining for insulin, Pdx-1 or C/EBPβ positive cells, as well as total cell numbers. Cell counts were carried out as described in the Materials and methods section. Bars are standard deviations. Statistical differences are shown by ***$P < 0.001$ versus control (CTL), **$P < 0.01$ versus control *$P < 0.05$ versus control. (**J**) RIN-5F cells were cultured for 1 week with or without 1 μM Dex. The cells were prepared for Western blotting, as described in the Materials and methods section, and the blot was probed with anti-Pdx-1 and anti-α-tubulin antibodies. C/EBPβ protein was detected by immunoprecipitation and Western blotting, as described in the Materials and methods section. (**K**) The effect of Dex on insulin secretion. RIN cells were cultured for 5 days with or without 1 μM Dex. The measurement of glucose-stimulated insulin release from RIN cells was carried out using a rat insulin ELISA kit. Results are shown as means $\pm$ S.D. Statistical differences are shown by *$P < 0.05$ versus control ($n = 3$).

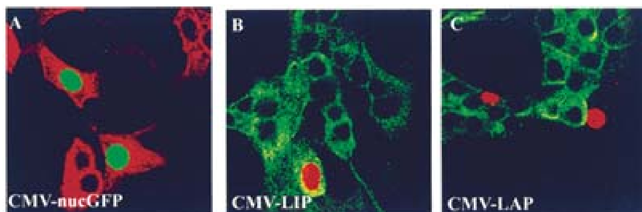

Figure 7 Regulation of insulin expression by C/EBPβ

RIN-5F cells were transfected with CMV-nucGFP, CMV-LIP (dominant-negative form of C/EBPβ) or CMV-LAP (active form of C/EBPβ). After 72 h of transfection, cells were fixed and dual stained with (**A**) anti-insulin (red)/anti-GFP (green) antibodies or anti-insulin (green)/anti-C/EBPβ (red) antibodies. LIP (**B**) or LAP (**C**) can be detected by immunohistochemistry because the C/EBPβ antibody is raised to the C-terminus. LIP or LAP transfected cells are intensely stained. Typical fields are shown.

is that Dex inhibits insulin synthesis by destabilizing preproinsulin mRNA [31]. While this effect of Dex has been known to exist in HIT cells, it remains to be tested in embryonic pancreatic buds.

In summary, we found that insulin expression is reduced in offspring from Dex-treated rats and the *in vitro* studies suggest that the mechanism involves permanent damage to β-cells arising from early expression of C/EBPβ. So, it is possible that at least a part of the fetal programming effect can be explained as follows. Stress to the mother elevates circulating glucocorticoids and/or placental 11β-HSD2 is deficient, allowing excess glucocorticoids in the fetus. This elevates expression of C/EBPβ in the developing pancreas, which suppresses the late phase of expression of Pdx-1, necessary for β-cell differentiation, and also antagonizes the transcription of the *insulin* gene itself. The net effect is a permanent reduction of β-cell capability to produce insulin. In later life, other factors [3], such the intrinsic insulin resistance of this model [32], perhaps amplified by obesity, increase the demand on the pancreatic islets which they are unable to meet, hence leading to type 2 diabetes.

This work was funded by the Medical Research Council (D.T. and J.M.W.S.), the Royal Society (D.T.) and the Wellcome Trust (J.R.S.). We also thank Dr Cheryl Docherty for help with *in vivo* studies.

© 2003 Biochemical Society

REFERENCES

1 Barker, D. J. P., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A. and Robinson, J. S. (1993) Fetal nutrition and cardiovascular disease in adult life. Lancet **341**, 938–941

2 Edwards, C. R. W., Benediktsson, R., Lindsay, R. S. and Seckl, J. R. (1993) Dysfunction of placental glucocorticoid barrier – link between fetal environment and hypertension. Lancet **341**, 355–357

3 Holness, M. J., Langdown, M. L. and Sugden, M. C. (2000) Early-life programming of susceptibility to dysregulation of glucose metabolism and the development of Type 2 diabetes mellitus. Biochem. J. **349**, 657–665

4 Campbell, A. L. and Murphy, B. E. P. (1977) The maternal-fetal cortisol gradient during pregnancy and at delivery. J. Clin. Endocrinol. Metab. **45**, 435–440

5 Benediktsson, R., Calder, A. A., Edwards, C. R. and Seckl, J. R. (1997) Placental 11 β-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. Clin. Endocrinol. **46**, 161–166

6 Reinisch, J. M., Simon, N. G., Karwo, W. G. and Gandleman, R. (1978) Prenatal exposure to prednisone in humans and animals retards intra-uterine growth. Science **202**, 436–438

7 Seckl, J. R., Cleasby, M. and Nyirenda, M. J. (2000) Glucocorticoids, 11β hydroxysteroid dehydrogenase, and fetal programming. Kidney Int. **57**, 1412–1417

8 Benediktsson, R., Lindsay, R., Noble, J., Seckl, J. R. and Edwards, C. R. W. (1993) Glucocorticoids exposure *in utero*: a new model for adult hypertension. Lancet **341**, 339–341

9 Levitt, N. S., Lambert, E. V., Woods, D., Hales, D., Andrew, R. and Seckl, J. R. (1996) Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology **64**, 412–418

10 Lindsay, R. S., Lindsay, R. M., Edwards, C. R. and Seckl, J. R. (1996) Inhibition of 11-β-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring. Hypertension **27**, 1200–1204

11 Lindsay, R. S., Lindsay, R. M., Edwards, Waddell, B. and Seckl, J. R. (1996) Programming of glucose tolerance in the rat: role of placental 11β-hydroxysteroid dehydrogenase. Diabetologia **39**, 1299–1305

12 Ryffel, G. U. (2001) Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences J. Mol. Endocrinol. **27**, 11–29

13 Slack, J. M. W. and Tosh, D. (2001) Transdifferentiation and metaplasia – switching cell types. Curr. Opin. Genetics Dev. **11**, 581–586

14 Tosh, D. and Slack, J. M. W. (2002) How cells change their phenotype. Nat. Rev. Mol. Cell Biol. **3**, 187–194

15 Shen, C.-N., Slack, J. M. W. and Tosh, D. (2000) Molecular basis of transdifferentiation of pancreas to liver. Nat. Cell Biol. **2**, 879–887

16 Tosh, D., Shen, C.-N. and Slack, J. M. W. (2002) Differentiated properties of hepatocytes induced from pancreatic cells. Hepatology **36**, 535–543

17 Tosh, D., Shen, C.-N. and Slack, J. M. W. (2002) Conversion of pancreatic cells to hepatocytes. Biochem. Soc. Trans. **30**, 51–54

18 Percival, A. C. and Slack, J. M. W. (1999) Analysis of pancreatic development using a cell lineage label. Exp. Cell Res. **247**, 123–132

19 Horb, L. D. and Slack, J. M. W. (2000) Role of cell division in branching morphogenesis and differentiation of the embryonic pancreas. Int. J. Dev. Biol. **44**, 791–796

20 Nyirenda, M. J., Lindsay, R. S., Kenyon, C. J., Burchell, A. and Seckl, J. R. (1998) Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. J. Clin. Invest. **101**, 2174–2181

21 Slack, J. M. W. (1995) Developmental biology of the pancreas. Development **121**, 1569–1580

22 Seckl, J. R. (2001) Glucocorticoids, programming of the fetus; adult phenotypes and molecular mechanisms. Mol. Cell. Endocrinol. **185**, 61–71

23 Blondeau, B., Lesage, J., Czernichow, P., Dupouy, J. P. and Breant, B. (2001) Glucocorticoids impair fetal β-cell development in rats. Am. J. Physiol. Endocrinol. Metab. **281**, E592–E599

24 Blondeau, B., Avril, I., Duchene, B. and Breant, B. (2002) Endocrine pancreas development is altered in foetuses from rats previously showing intra-uterine growth retardation in response to malnutrition. Diabetologia **45**, 394–401

25 Langley-Evans, S. C., Phillips, G. J., Benediktsson, R., Gardner, D. S., Edwards, C. R., Jackson, A. A. and Seckl, J. R. (1996) Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. Placenta **17**, 169–172

26 Jonsson, J., Carlsson, L., Edlund, T. and Edlund, H. (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature (London) **371**, 606–609

27 Ohlsson, H., Karlsson, K. and Edlund, T. (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J. **12**, 4251–4259

28 Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K. and Edlund, H. (1998) Beta-cell-specific inactivation of the mouse Ipf1/Pdx-1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev. **12**, 1763–1768

29 Sharma, S., Jhala, U. S., Johnson, T., Ferreri, K., Leonard, J. and Montminy, M. (1997) Hormonal regulation of an islet-specific enhancer in the pancreatic homeobox gene STF-1. Mol. Cell Biol. **17**, 2598–2604

30 Lu, M., Seufert, J. and Habener, J. F. (1997) Pancreatic β-cell-specific repression of insulin gene transcription by CCAAT/Enhancer-binding protein β J. Biol. Chem. **272**, 28349–28359

31 Philippe, J. and Missotten, M. (1990) Dexamethasone inhibits insulin biosynthesis by destabilizing insulin messenger ribonucleic acid in hamster insulinoma cells. Endocrinology **127**, 164–1645

32 Cleasby, M. E., Kelly, P. A., Walker, B. R. and Seckl, J. R. (2003) Programming of rat muscle and fat metabolism by *in utero* overexposure to glucocorticoids. Endocrinology **144**, 999–1007

Received 20 January 2003/12 June 2003; accepted 24 June 2003
Published as BJ Immediate Publication 26 June 2003, DOI 10.1042/BJ20030140

© 2003 Biochemical Society