Disrupted Pancreatic Exocrine Differentiation and Malabsorption in Response to Chronic Elevated Systemic Glucocorticoid

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Glucocorticoids are antiinflammatory therapeutics that have potent effects on cell differentiation. The aim of this study was to establish whether systemic glucocorticoid exposure significantly affects pancreatic differentiation in vivo because hepatocyte-like cells have been documented to occur in the diseased rodent pancreas. Expression of hepatic markers was examined in pancreata from mice genetically modified to secrete elevated circulating endogenous glucocorticoid [Tg(Crh)]. Tg(Crh) mice with elevated glucocorticoid appeared cushingoid and by 21 weeks of age were obese, insulin-resistant, and had extensive areas of hepatic gene expression in exocrine tissue. Acinar cells from Tg(Crh) mice costained for both amylase and cyp2e1, suggesting direct acinar-hepatic transdifferentiation. Hepatic expression increased with age in the pancreas to such an extent that malabsorption and rapid weight loss occurred in a subset of aging mice; this effect was reversed by dietary porcine pancreatic enzyme supplementation. Indeed, pancreatic expression of hepatic markers was prevented by adrenalectomy, establishing a direct role for glucocorticoid. Elevated levels of circulating glucocorticoid therefore promote a transdifferentiation of adult exocrine pancreas into hepatocyte-like cells, and chronic exposure results in pancreatic malfunctions. Glucocorticoids are thus capable of modulating the differentiation of terminally differentiated adult cells. (Am J Pathol 2010, 177:1225–1232; DOI: 10.2353/ajpath.2010.100107)
ers, suggesting that acinar cells may also transdifferentiate into hepatocyte-like cells in vivo in response to elevated glucocorticoid.

The effects of elevated glucocorticoid exposure have therefore been examined in pancreata from a transgenic mouse that secretes elevated levels of endogenous glucocorticoid. We show for the first time that sustained mouse that secretes elevated levels of endogenous glucocorticoid.

Table 1. DNA Oligonucleotide Sequences Used in RT-PCR or PCR Genotyping

<table>
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<tr>
<th>RT-PCR</th>
<th>Sequence</th>
<th>Annealing conditions</th>
<th>Comments</th>
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<tr>
<td>mcyp2eUS</td>
<td>5′-GTTTCGAGGAAATCTATCCATC-3′</td>
<td>43°C</td>
<td>Will amplify mouse cyp2e1 (NM_021282.2) cDNA sequence of 223 bp</td>
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<td>3′-AATGCTTACCGAGGATGT-5′</td>
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<td>Will amplify mouse cyp3a25 (NM_019792.1) cDNA sequence of 374 bp</td>
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<td>58°C</td>
<td>Will amplify mouse CPS-I (NM_001080809.1) cDNA sequence of 285 bp</td>
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<tr>
<td>mcyps-IDS</td>
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<td>55°C</td>
<td>Will amplify mouse albumin (NM_009654.3) cDNA sequence of 354 bp</td>
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<td>malbuminUS</td>
<td>5′-TGACTGAGCTGCTGCTGCTG-3′</td>
<td>55°C</td>
<td>Will amplify mouse pancreatic amylase 2a2 (NM_001160152.1), 2a3 (NM_001160151.1), 2a4 (NM_001160150.1), and 2a5 (NM_001042711.2) cDNA sequences of 241 bp</td>
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<td>mhGAPDHUS</td>
<td>5′-TGACATCCAAAGGAGGTGAGG-3′</td>
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<td>mhGAPDHDS2</td>
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<td>Will amplify rat (NM_053357) and mouse (NM_007614) cDNA sequences of 187 bp</td>
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<td>Will amplify rat (NM_031019.1) cDNA sequence of 202 bp</td>
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Materials and Methods

Animal Studies

C57Bl6 Tg(Crh) transgenic mice, originally generated by the laboratory of Vale et al18 (in which expression of the rat corticotrophin releasing factor under control of the mouse metallothionein promoter and human growth hormone 3′ untranslated region results in elevations in circulating glucocorticoid), were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in individually ventilated cages at stocking densities according to UK Home Office Codes of Practice, with sawdust bedding (Gold chip, BS and S Ltd, Edinburgh, Scotland) and nesting material (Shredded paper, DBM, Broxburn, Scotland). The animal rooms were maintained at 23°C ± 1°C, 48% humidity, and on a 12/12-hour light/dark cycle (lights on at 07:00). Food (CRM (P), SDS Ltd, Essex, England) and water were provided ad libitum. Female adult mice were used in all studies (because only males could be used for successful mating with wild-type females) with genotypes checked by PCR using IMR0135 (US) and IMR0136 (DS) primers and conditions (see Table 1). All mice were on a C57Bl6 background. The mice showed overt clinical signs of Cushing’s syndrome within 15 weeks of age (eg, hair loss, obesity, thinning skin).

To test for glucose tolerance, mice were starved overnight for 9 hours before injection of 2g glucose/kg body weight by i.p. injection. Serum was prepared from tail vein bleeds just before and at various times after glucose injection. Insulin levels were determined using a murine insulin ELISA kit obtained from Millipore (Watford, UK). Glucose was determined by enzymatic assay as previously described.

Some mice (between 8 and 10 weeks of age) underwent adrenalectomy or a sham procedure. Mice were anesthetized with isoflurane (5% induction, 2–2.5% maintenance) in 100% oxygen. Bilateral adrenalectomy, or a sham procedure, was performed using a dorsal approach. Buprenorphine (0.1 mg/kg body weight s/c) and/or Meloxicam (5 mg/kg body weight s/c) was administered to provide postsurgical analgesia. Mice were allowed to recover in a warmed environment, and adrenalectomized mice were provided with a salt lick ad libitum.

Porcine pancreatic enzyme (Creon, Solvay) was added to powdered diet and provided to mice ad libitum at no more than 4000 units per mouse per day. The use of animals in this project was approved by the local Ethical Committee and the UK Home Office.
**Immunohistochemistry**

Tissues were fixed in formalin and processed for immunohistochemistry as previously outlined. Antibodies were obtained from local commercial suppliers as previously reported except anti-cyp3a25, which was generously provided by Dr. Rob Edwards (Imperial College, London, UK).

**RT-PCR**

Total RNA was purified using Trizol (Invitrogen, Paisley, UK) and RT-PCR performed and analyzed as previously outlined (primer sequences are given in Table 1).

**Western Blotting**

Western blotting was performed as previously outlined with antibodies outlined as above. Detection was achieved using an ECL kit (Amersham, UK).

**Cell Isolation and Culture**

Rat hepatocytes were prepared by collagenase perfusion essentially as previously described. B-13 cells were routinely cultured in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 80 u/ml penicillin, and 80 µg/ml streptomycin, under which conditions the cells remained proliferative and phenotypically stable. B-13 cells were subcultured using trypsin at a ratio of 3:1 by standard methods. All cells were incubated at 37°C in an humidified incubator gassed with 5% CO₂ in air. Dexamethasone (DEX), human corticotropin releasing hormone (CRH, identical to rat sequence except for an additional N-terminal 3 amino acids), and adrenocorticotropic hormone (ACTH, a truncated 1-24 peptide sequence conserved between human, rat, and mouse) was used, which shows 85% activity of the full length peptide sequence) were all purchased from the Sigma Chem Co. (Poole, UK) and were added to medium from 1000-fold concentrated ethanol vehicle solvated stocks. Control cells were treated with ethanol alone.

**Statistics**

The Student’s t-test (two-tailed) was used to test for replicate statistical significance.

**Results**

Tg(Crh) Mice Show Symptoms of Chronic Elevated Systemic Glucocorticoid

Endogenous glucocorticoid secretion is circadian and ultradian, which results in rapid changes in concentration in the circulating blood that is experimentally complex to model. To examine the effect of glucocorticoid on pancreatic differentiation pancreata from transgenic mice expressing rat CRH under control of the metallothionein-1 promoter were examined. These mice have high circulating glucocorticoid, and from around 15 weeks of age the Tg(Crh) mice showed overt signs of elevated circulating glucocorticoid, including thymic and splenic atrophy, alopecia, and reduced skin thickness (Figure 1 and data not shown). These mice therefore exhibit many of the clinical symptoms of Cushing’s disease observed in humans.

Initial examination at 21 weeks of age showed that Tg(Crh) mice were obese (Figure 1A) with body weights significantly greater than age-matched wild-type animals (Figure 1B). The relative weights of several organs were significantly lower—most notably the pancreas, which was reduced by 64% in Tg(Crh) compared with wild-type mice (Figures 1, C and D). In contrast, relative liver weights were significantly increased in Tg(Crh) mice (Fig-
ure 1D), likely due in part to hepatic steatosis (Figure 2A).

Relative visceral fat weight were also significantly increased in Tg(Crh) mice (Figure 1D). Examination of pancreata in 21-week-old Tg(Crh) and wild-type mice showed that Tg(Crh) mice had significant pancreatic islet hyperplasia (Figure 2, A and B), a recognized response to high levels of glucocorticoids.22 Immunohistochemical staining of pancreata indicated that the islets from Tg(Crh) were positive for insulin (Figure 2B), indicating marked cell proliferation. Immunoquantification of insulin levels confirmed that the Tg(Crh) pancreas contained significantly more insulin (Figure 2C). Despite similar circulating glucose levels, there were significantly higher serum levels of insulin in unstarved Tg(Crh) mice (Table 2). Glucose tolerance tests showed that Tg(Crh) mice took longer to reduce the serum levels of glucose after i.p. injection of glucose (Figure 2D). Tg(Crh) mice were therefore insulin-resistant at 21 weeks of age.

Widespread Age-Dependent Appearance of Hepatocytic Tissue in Acinar Pancreata from Tg(Crh) Mice

To determine whether elevated circulating glucocorticoid affected acinar pancreatic differentiation, tissue sections from 21-week-old mice were initially screened for cyp2e1 expression. Figure 3A shows that pancreata from Tg(Crh) mice had widespread areas of acinar tissue that stained positive for cyp2e1, whereas the tissue was negative in wild-type mice. Islet cells have been reported to express some isoforms of cytochrome P450s.23 However, under the staining conditions used and by using liver as a positive control in all staining procedures, cyp2e1 expression was not detectable in islet tissue (eg, see Figure 3A, lower middle panel, also Figure 3B). Costaining tissue for amylase and cyp2e1 demonstrated that acinar tissue from Tg(Crh) mice stained positive for both markers but only for amylase in acinar tissue from wild-type mice (Figure 3B). Cells were isolated from Tg(Crh) and wild-type pancreata and costained for amylase and cyp2e1 expression to more effectively quantitate the number of pancreas cells staining positive for cyp2e1. Acinar cells from Tg(Crh) mice stained for either amylase only (39 ± 6.4%), or amylase and cyp2e1 (56 ± 7.6%). More rarely, acinar-like cells stained for cyp2e1 only.

### Table 2. Serum Glucose and Insulin Levels in Wild-Type and Tg(Crh) Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Serum glucose (mmol/L)</th>
<th>Serum insulin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl6 wild-type</td>
<td>7.0 ± 0.31</td>
<td>1.4 ± 0.11</td>
</tr>
<tr>
<td>C57Bl6 Tg(Crh)</td>
<td>6.8 ± 0.42</td>
<td>2.6 ± 0.30*</td>
</tr>
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</table>

Blood was collected from 21-week-old mice by tail vein collection at 10:00 AM. Serum was prepared and analyzed as outlined in experimental section. Data are the mean and standard deviation of at least four animals per group.

*P < 0.05 compared to wild-type using Student’s t-test (two-tailed).
Figure 3. Widespread expression of hepatic markers in adult (21-week-old) Tg(Crh) mouse pancreas. A: Liver and pancreas sections from adult mice immunostained for cyp2e1. ‘No 1st Ab,’ sections stained without the addition of the primary (CYP2E) antibody. B: Pancreata were costained for amylase (green) and cyp2e1 (red) and expression examined by fluorescence microscopy with DAPI staining (blue) to identify nuclei. No primary antibody staining was used as controls (not shown), and all microscope/software settings retained for all Figures. I, islet. C: Wild-type female mice were mated with male Tg(Crh) mice and fetuses harvested at approximately 14 days gestation. DNA was isolated from each fetus for genotyping (lower panel) and RNA was isolated from pancreata (and brain to check for transgene expression; see Table 1). Expression of the indicated transcript was determined by RT-PCR and compared with wild-type and Tg(Crh) pancreas and liver expression. D: Western blot of homogenized pancreata prepared from wild-type and Tg(Crh) mice at the indicated age. Twenty micrograms of total protein were loaded in each lane and expression for the indicated marker determined. E: RT-PCR analysis from RNA isolated from liver and pancreas tissues and screened for the indicated transcript—30 cycles for all samples. F: Western blot of homogenized tissues prepared from wild-type and Tg(Crh) mice. Twenty micrograms of total protein were loaded in each lane and expression for the indicated marker determined. Results for all panels are typical of at least four animals per time point and genotype.

Figure 3, C and D indicates that the appearance of hepatocyte-like cells in the pancreata of Tg(Crh) mice was an adult-specific response, because hepatic mRNA transcript expression was only observed in adult and not fetal Tg(Crh) pancreata (Figure 3C) and protein levels detectable only in Tg(Crh) pancreata from between 4 and 21 weeks of age (Figure 3D). The lack of hepatic gene expression in fetal pancreata occurred despite evidence
of rat CRH transgene expression in fetal Tg(Crh) brain (see supplemental Figure 1 at http://ajp.amjpathol.org).

The expression of cyp2e1 in Tg(Crh) pancreata therefore appears to be a response to long-term elevated glucocorticoid exposure in the adult.

Figure 3, E and F demonstrates that the appearance of hepatocyte markers in the pancreata from Tg(Crh) mice was not restricted to cyp2e1 but that a range of liver-specific and liver-enriched genes were detected at the mRNA level (Figure 3E) and protein level (Figure 3F). The quantitative nature of Western blotting supports the immunohistochemical data and demonstrates that a significant proportion of the Tg(Crh) pancreata were expressing hepatic levels of genes, and that the extent of exocrine pancreas transdifferentiation may have significant metabolic consequences.

The Appearance of Hepatocyte-Like Cells in Tg(Crh) Pancreata Is Dependent on Elevated Glucocorticoid

Adrenalectomising Tg(Crh) mice at 8–10 weeks of age (before the appearance of liver marker gene expression in the pancreas) prevented the distinctive appearance (Figure 1A) seen with Tg(Crh) by 21 weeks of age (data not shown), the changes in relative organ weight (see supplemental Figure 2 at http://ajp.amjpathol.org), and the expression of hepatic markers in the Tg(Crh) pancreas (Figure 4A), indicating an absolute dependence on elevated glucocorticoid in vivo (and that the response is not associated with elevated rat CRH transgene expression or elevated ACTH expression). This observation is supported by the lack of response in the B-13 pancreatic acinar cell line to CRH and ACTH hormones (Figure 4B). The B-13 cell transdifferentiates into hepatocyte-like cells in response to glucocorticoid exposure via suppression of WNT signaling activity and C/EBP-β induction.17,21,24,25 Figure 4B indicates that the B-13 cell responds to the glucocorticoid dexamethasone but not to CRH and ACTH.

The Appearance of Hepatocyte-Like Cells in Tg(Crh) Pancreata Is Associated with Malabsorption and Weight Loss as Animals Age

As indicated in Figure 1, Tg(Crh) mice were obese and had significantly greater body weights at 21 weeks of age compared with age-matched wild-type mice. However, as Tg(Crh) mice aged, they began to lose weight, with a subpopulation (approximately 40%, denoted as Tg(Crh)*) losing body weight comparatively rapidly post 32 weeks of age (Figure 5A). Between 32 and 42 weeks of age of, both Tg(Crh) and Tg(Crh)* mice body weights were significantly lower than wild-type mice, whereas their weights were significantly greater or similar, respectively, at 32 weeks (Figure 5B). Figure 5C demonstrates that the relative levels of amylase expression in pancreata from Tg(Crh) mice were lower than the levels in wild-types and markedly lower in pancreata from Tg(Crh)* mice, which had lost the most weight. It was therefore hypothesized that body weight losses in Tg(Crh) mice were associated with a loss of exocrine pancreatic function and reduced ability to digest and absorb nutrients from the gut. To test this hypothesis, mouse diet was supplemented with enteric-coated porcine pancreatic enzyme and body weights monitored for 40 days. Figure 5, D and E supports the hypothesis of exocrine pancreatic dysfunction and malabsorption because wild-type animals (with functional exocrine pancreas) were not affected by pancreatic enzyme supplementation, whereas both Tg(Crh) and Tg(Crh)* mice gained significant body weight.

Discussion

It has been known for some time that hepatocyte-like cells appear in the rodent pancreas in response to damage or ectopic expression of growth factors.1–4 This article demonstrates for the first time that this response also
occurs in response to prolonged elevated glucocorticoid exposure and in the absence of any overt pancreatic damage. The primary theory proposed for the presence of hepatocyte-like cells in rodent pancreas is that a pancreatic progenitor cell—likely in the ductal regions—aberrantly differentiates into hepatocyte-like cells because the preceding tissue damage leads to an altered extracellular matrix and/or growth factor environment and subsequent misdirected differentiation of progenitor cells.

Short-term administration of glucocorticoid to rats gives rise to occasional acinar cells that stain positive for hepatic markers in pancreata with no evidence of pancreatic damage. However, this observation could be the result of a relatively rare response of little biological relevance. The widespread hepatic expression in Tg(Crh) pancreata demonstrate that this response is a common pathophysiological response to elevated glucocorticoid and therefore of both clinical interest (for patients maintained on long term systemic glucocorticoid therapy) and biological interest (because it demonstrates a role for glucocorticoid on cellular differentiation in vivo). The Tg(Crh) mouse model and rat glucocorticoid dosing model support the concept that hepatocyte-like cells are emerging primarily by transdifferentiation of acinar cells—hepatocyte-like cells appear in regions with no apparent associated ductal link and express both acinar (amylase) and hepatic (cyp2e1) genes. Indeed, the process in vivo appears to mirror the transdifferentiation of B-13 cells into hepatocytes in response to glucocorticoid,21,24,25 suggesting that the B-13 cell is modeling a pathophysiological response of acinar cells to glucocorticoid in vivo.

A common adverse side effect of glucocorticoid therapy is infection because of the antiinflammatory properties of steroids. A screen of Drug Analysis Prints [which list all UK spontaneous suspected adverse drug reactions reported through the Yellow Card Scheme to the MHRA and the UK Government’s independent scientific committee on medicines safety, the Commission on Human Medicines (CHM)] between July 1, 1963 and November 26, 2009 indicates that the number of adverse events categorized as “metabolic” (eg, diabetes mellitus, diabetic complications, hyperglycemia, pancreatitis) for dexamethasone, betamethasone, and prednisolone occur at 60.0%, 50.0%, and 36.3%, respectively, as often as infection. The level of metabolic adverse effects to glucocorticoids is therefore significant within the context of adverse effects of glucocorticoids and exocrine trans-
Differentiation may be a significant, yet unrealized, contribution to this. These data therefore predict that long-term systemic glucocorticoid therapy may have significant adverse effects on pancreatic exocrine function that may progress to malabsorption. This should be a consideration when clinicians are prescribing long-term systemic glucocorticoid therapy.

References


