A primary defect in glucose production alone cannot induce glucose intolerance without defects in insulin secretion

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Abstract

Increased glucose production is associated with fasting hyperglycaemia in type 2 diabetes but whether or not it causes glucose intolerance is unclear. This study sought to determine whether a primary defect in gluconeogenesis (GNG) resulting in elevated glucose production is sufficient to induce glucose intolerance in the absence of insulin resistance and impaired insulin secretion. Progression of glucose intolerance was assessed in phosphoenolpyruvate carboxykinase (PEPCK) transgenic rats, a genetic model with a primary increase in GNG. Young (4–5 weeks of age) and adult (12–14 weeks of age) PEPCK transgenic and Piebald Virol Glaxo (PVG/c) control rats were studied. GNG, insulin sensitivity, insulin secretion and glucose tolerance were assessed by intraperitoneal and intravascular substrate tolerance tests and hyperinsulinaemic/euglycaemic clamps. Despite elevated GNG and increased glucose appearance, PEPCK transgenic rats displayed normal glucose tolerance due to adequate glucose disposal and robust glucose-mediated insulin secretion. Glucose intolerance only became apparent in the PEPCK transgenic rats following the development of insulin resistance (both hepatic and peripheral) and defective glucose-mediated insulin secretion. Taken together, a single genetic defect in GNG leading to increased glucose production does not adversely affect glucose tolerance. Insulin resistance and impaired glucose-mediated insulin secretion are required to precipitate glucose intolerance in a setting of chronic glucose oversupply.

Journal of Endocrinology (2011) **210,** 335–347

Introduction

Type 2 diabetes (T2D) is characterised by glucose intolerance that is contributed to by both defects in insulin action (in liver and muscle/fat) and insulin secretion. Whether defects in both insulin action and secretion are necessary for glucose intolerance to develop is still debatable (Valera *et al.* 1994, Trinh *et al.* 1998, Sun *et al.* 2002, Visinoni *et al.* 2008). We have previously hypothesised that a primary defect in the suppression of gluconeogenesis (GNG) resulting in inappropriately elevated endogenous glucose production (EGP; Lamont *et al.* 2003), will cause glucose intolerance and fasting hyperglycaemia, as observed in T2D (Consoli *et al.* 1989, Nurjhan *et al.* 1992, Perriello *et al.* 1997, Boden *et al.* 2001). Although GNG occurs predominantly in the liver, it has been suggested that the kidney accounts for ∼40% of overall GNG (Stumvoll *et al.* 1998, Cersosimo *et al.* 2000, Gerich *et al.* 2001). Insulin exerts its inhibitory actions by negatively regulating the transcription of the rate-determining enzymes phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase). Elevated GNG, stemming from impaired insulin-mediated suppression of gluconeogenic enzyme transcription is a defect observed in both animals and humans with glucose intolerance and T2D (Chang & Schneider 1970, Seidman *et al.* 1970, Andrikopoulos & Proietto 1995, Arizmendi *et al.* 1999, Gastaldelli *et al.* 2000, Roden *et al.* 2000, Song *et al.* 2001, Chevalier *et al.* 2006).

To directly test whether an increase in EGP can lead to glucose intolerance and hyperglycaemia, we and others have generated genetic rodent models in which the key gluconeogenic enzymes have been overexpressed in the liver and/or kidneys (Valera *et al.* 1994, Trinh *et al.* 1998, Sun *et al.* 2002, Visinoni *et al.* 2008). Specifically, transgenic models in which PEPCK has been overexpressed have been characterised by glucose intolerance and insulin resistance in both liver and muscle/fat (Valera *et al.* 1994, Sun *et al.* 2002). Similarly, a transgenic mouse in which G6Pase was over-expressed using adenovirus technology also displayed glucose intolerance and insulin resistance (Trinh *et al.* 1998). From these studies one could conclude that a primary elevation in GNG can cause glucose intolerance and insulin resistance. However, this relationship is challenged when one considers that not all models of increased GNG develop this abnormality. For example, we have generated a liver-specific FBPase transgenic mouse that is characterised by normal

DOI: 10.1530/JOE-11-0126
0022–0795/11/0210–335
Online version via http://www.endocrinology-journals.org

glucose tolerance and plasma glucose concentrations (Lamont *et al.* 2006). This inconsistency raises the possibility that a single genetic defect leading to elevated GNG will not necessarily lead to glucose intolerance. From the studies in rodent models genetically geared towards glucose overproduction outlined above, it is unclear whether additional factors are required to induce glucose intolerance. For example, none of these investigations have directly assessed the impact of a primary elevation in GNG on insulin secretion. Therefore, it is not clear whether an initial defect in GNG alone will cause glucose intolerance and hyperglycaemia.

Therefore, this study sought to determine whether a primary increase in GNG was sufficient to induce glucose intolerance in the absence of insulin resistance and impaired insulin secretion. We hypothesise that an increase in EGP is not sufficient to cause glucose intolerance and that defects in insulin action and/or secretion also need to occur. To this end, we examined glucose tolerance, glucose-mediated insulin secretion, GNG (hepatic, renal and total), EGP and insulin sensitivity in young and adult PEPCK transgenic and Piebald Virol Glaxo (PVG/c) control rats. The results showed that a primary increase in glucose production did not lead to glucose intolerance, due to normal hepatic and peripheral insulin sensitivity and robust glucose-mediated insulin secretion. In contrast, acquired insulin resistance (both hepatic and peripheral) and defects in glucose-mediated insulin secretion, disturbed the equilibrium between glucose output and utilisation and resulted in glucose intolerance, mild hyperglycaemia and hyperinsulinaemia.

Materials and Methods

Animals

PEPCK transgenic rats on the PVG/c background strain expressing the PEPCK gene in the liver and kidney under the control of the metallothionein promoter were produced as described previously (Rosella *et al.* 1995). Rats were housed in the Austin Health BioResources Facility at the Heidelberg Repatriation Hospital. Lighting was artificial and timer controlled with a 12 h light:12 h darkness cycle. Room temperature was kept constant at 22 °C. Rats were fed a standard laboratory chow diet (Ridley AgriProducts, Pakenham, VIC, Australia) and water, made available *ad libitum*. All procedures were approved by the Austin Health Animal ethics committee (A2003/01822 and A2007/03057). For each of the studies described below, young (4–5 weeks of age) and adult (12–14 weeks of age) male and female PEPCK transgenic and PVG/c control rats were used.

Energy balance measurements

Age-matched PEPCK transgenic and PVG/c control rats were housed individually and given a 3-day acclimatisation period before the commencement of the studies. Body weights were measured daily at 0900 h over 1 week ($n=8$–13 PVG/c; $n=8$–13 PEPCK). Weight of food given was recorded at 0900 h and daily food intake in grams was measured 24 h later ($n=5$ PVG/c; $n=5$ PEPCK).

Spontaneous physical activity was assessed in a subgroup of young and adult PEPCK and PVG/c rats ($n=4$ PVG/c; $n=4$ PEPCK) using a single cage animal activity monitor (Columbus Instruments, Columbus, OH, USA) as described previously (Funkat *et al.* 2004, Fam *et al.* 2007). Resting energy expenditure (REE), fat oxidation (Fox) and glucose oxidation (Gox) were measured in a subgroup of young and adult PEPCK and PVG/c rats ($n=3$–4 PVG/c; $n=3$–6 PEPCK) using indirect calorimetry as previously described and calculated (Thorburn *et al.* 1990, Fam *et al.* 2007).

Adiposity levels were determined by excision of selected white adipose tissue depots (subcutaneous, infrarenal and gonadal) and weighed separately at the conclusion of each of the studies below.

Surgery for basal turnover and hyperinsulinaemic/euglycaemic clamps

Following an overnight fast, rats were anaesthetised with an i.p. injection of sodium pentobarbitone (60 mg/kg) and heparinised saline (25 U/ml) filled polyethylene catheters (Critchley Electrical, Castle Hill, NSW, Australia) were inserted into the right jugular vein and left carotid artery. The venous catheter was used for infusion and the carotid catheter for blood sampling. A tracheostomy was performed to prevent upper airway obstruction. Body temperature was maintained at 37 °C and monitored throughout with an anal temperature probe. Anaesthesia was adequately maintained throughout the procedures.

Tracer infusions

For both basal turnover and hyperinsulinaemic/euglycaemic clamps, all rats were infused with an initial 2 min priming dose of radio-labelled glucose tracer [6-^{3}H]glucose at a rate of 100 μBq/min in 0·9% saline followed by a constant infusion of tracer at a rate of 5·5 μBq/min in 0·9% saline for the duration of the experiment (155 min in total) as previously described by our laboratory (Lamont *et al.* 2003, Visinoni *et al.* 2008).

Basal turnover, hyperinsulinaemic/euglycaemic clamps and glucose uptake into peripheral tissues

Basal turnover ($n=10$ PVG/c; $n=8$ PEPCK), hyperinsulinaemic/euglycaemic clamps ($n=5$–6 PVG/c; $n=4$–6 PEPCK) and peripheral glucose uptake into individual tissues under clamp ($n=5$–6 PVG/c; $n=4$–6 PEPCK) conditions were performed in subgroups of PVG/c control and PEPCK transgenic rats following an overnight fast as previously described by our laboratory and others

(Kraegen *et al.* 1991, Nolan & Proietto 1994, Lamont *et al.* 2003). For the hyperinsulinaemic/euglycaemic clamp, insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was infused during the clamp at a dose of 2 mU/kg for PEPCK transgenic rats and 4 mU/kg for PVG/c control rats as previously reported (Lamont *et al.* 2003) to produce similar plasma insulin concentrations in the two groups of rats. Blood glucose was maintained at euglycaemia with a 5% glucose solution. Plasma samples for basal turnover and the hyperinsulinaemic/euglycaemic clamp were taken at 90, 100 and 110 min. Peripheral glucose uptake was performed immediately following the 110 min blood sample from the hyperinsulinaemic/euglycaemic clamps. A bolus of 2-[1-^{14}C]deoxyglucose (370 μBq) was injected intravascularly and blood samples collected at 2, 5, 10, 15, 30 and 45 min thereafter. Immediately following the final 45 min blood samples, rats were overdosed with sodium pentobarbitone (60 mg/kg) and white adipose tissue, brown adipose tissue and white quadriceps excised immediately, snap frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

Rates of GNG

Basal rate of GNG from alanine and glutamine following an overnight fast were determined as previously described by our group (Andrikopoulos & Proietto 1995, Visinoni *et al.* 2008). Rats (n=4–5 PVG/c; n=4–5 PEPCK) were administered with a priming bolus dose of [6-^{3}H]glucose (1·27 μCi) and a combination of [U-^{14}C]alanine/[U-^{14}C]glutamine (0·45 μCi) for 2 min before a constant infusion of 0·092 μCi/min [6-^{3}H]glucose and 0·032 μCi/min of the combined [U-^{14}C]alanine/[U-^{14}C]glutamine for 2 h.

For the determination of GNG under clamp conditions, rats (n=4–5 PVG/c; n=4–5 PEPCK) were subjected to an initial hyperinsulinaemic/euglycaemic clamp (as above) without the combined [U-^{14}C]alanine/[U-^{14}C]glutamine for 110 min. At 120 min, rats were infused with a priming bolus of insulin before continuous infusion of 0·092 μCi/min [6-^{3}H]glucose, 0·032 μCi/min of the combined [U-^{14}C]alanine/[U-^{14}C]glutamine and 4 mU/kg per min (0·45 μCi) for 2 h. Blood glucose was monitored every 5 min and euglycaemia maintained with a 25% glucose solution. Plasma samples from both tests were taken at 90, 100 and 110 min and treated as described below for glucose, alanine and glutamine assays.

Intraperitoneal tolerance tests

Intraperitoneal glucose tolerance tests (IPGTT) were performed as described previously (Lamont *et al.* 2006, Andrikopoulos *et al.* 2008), but modified for rats. Briefly, rats (n=10–15 PVG/c; n=10–15 PEPCK) were fasted overnight and anaesthetised with i.p. injection of sodium pentobarbitone (60 mg/kg). A blood sample (100 μl) was drawn from the tail vein and rats were injected i.p. with 1 g glucose/kg body weight. Subsequent blood samples (100 μl) were taken from the tail vein at 15, 30, 45, 60, 120 and 180 min for measurement of plasma glucose and insulin.

Intraperitoneal alanine tolerance tests (IPATT, 2 g/kg) were performed in overnight fasted, anaesthetised PEPCK transgenic and PVG/c control rats (n=6 PVG/c; n=5 PEPCK) at 4 weeks of age using the same protocol as with IPGTTs.

Intraperitoneal pyruvate tolerance tests were performed in rats (n=10 PVG/c; n=8 PEPCK), as described previously (Visinoni *et al.* 2008), following the estimation of GNG. Rats were then injected i.p. with sodium pyruvate (2 g/kg body weight, Sigma–Aldrich). Glucometer measurements of blood glucose were obtained every 15 min for 2 h.

Intravascular glucose tolerance

To determine insulin secretion we performed intravascular glucose tolerance tests (IVGTT) as described previously (Andrikopoulos *et al.* 2005, Aston-Mourney *et al.* 2007, Kebede *et al.* 2008), but modified for rats. Briefly, overnight fasted rats (n=10–11 PVG/c; n=12–15 PEPCK) were prepared for surgery as stated above. Rats were catheterised with a single heparinised saline (25 U/ml) filled polyethylene catheter into the left carotid artery for blood sampling. A basal blood sample was taken for collection of plasma. Glucose (1 g/kg body weight) was administered via the catheter and blood samples were taken at 2, 5, 10, 15, 30 and 45 min for measurement of plasma glucose and insulin concentrations.

Pair-feeding studies

PEPCK transgenic rats were pair-fed to the PVG/c control rats from 4 weeks of age for 12 weeks (n=5 PVG/c; n=5 PEPCK). Food intake was assessed in the control rats and this amount was given to the PEPCK transgenic rat the following week. Body weights were assessed every week to ensure that the PEPCK transgenic pair-fed rats matched the control animals. At the end of the 12-week feeding period, animals were fasted overnight and the IPGTT performed as described earlier.

Determination of whole-body glucose turnover (basal and clamp)

To determine whole-body glucose turnover, plasma samples collected at 90, 100 and 110 min were treated, processed and measured for the level of [6-^{3}H]glucose radioactivity as previously described by our laboratory (Nolan & Proietto 1994, Lamont *et al.* 2003). Briefly, a 25 μl aliquot of plasma was treated with equal volumes of 0·3 M barium hydroxide and 0·3 M zinc sulphate, centrifuged and 50 μl of supernatant passed through an anion exchange column (1·5 ml of Dowex 2X8-400 anion exchanger) to remove labelled lactate and pyruvate. Glucose was eluted from the column with 4 ml of milliQ water and 10 ml of scintillant (Ultima Gold, PerkinElmer, Waltham, MA, USA) added and radioactivity determined using a Packard 1900CA TriCarb liquid scintillation analyser (Packard, Meriden, CT, USA).

Determination of glucose uptake into individual tissues

To determine glucose uptake into muscle and fat tissues, the labelled 2-[1-^{14}C]deoxyglucose technique was used as previously described by our laboratory and others (Kraegen *et al*. 1991, Nolan & Proietto 1994, Lamont *et al*. 2003).

Determination of rates of GNG

To determine the rates of GNG, plasma samples were treated, processed and measured by methods previously described by our laboratory (Andrikopoulos & Proietto 1995).

Glucose, glutamine and alanine assays

The glucose, glutamine and alanine assays were performed as previously described by our laboratory (Andrikopoulos & Proietto 1995).

Plasma analytes

The concentration of circulating glucose was measured by the glucose oxidase assay using a GM7 Analox glucose analyser (Helena Laboratories, Mount Waverley, VIC, Australia). Plasma insulin concentrations were measured using a sensitive rat insulin RIA that cross-reacts 100% with human insulin (Linco Research, St Charles, MO, USA). Plasma leptin concentrations were measured using a rat leptin RIA (Linco Research).

Kidney and liver mRNA expression of gluconeogenic genes

Total RNA was extracted from kidney ($n=6$ PVG/c; $n=6$ PEPCK) and liver ($n=6$ PVG/c; $n=6$ PEPCK) using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA (1 μg) was DNAse treated using DNA-*free* (Ambion, Austin, TX, USA) before being reverse transcribed using random priming with an AMV reverse transcriptase kit (Promega). Relative expression of PEPCK, FBPase and G6Pase mRNA were measured by quantitative real-time PCR using pre-developed rat TaqMan Gene Expression Assays (Applied Biosystems, Scoresby, VIC, Australia) as described previously (Visinoni *et al*. 2008) using 18S as the endogenous control gene. The comparative C_t method ($\Delta\Delta C_t$) was used for relative quantification.

Statistical analysis

All data are presented as mean ± S.E.M. Comparisons between single parameters measured were made by the two-tailed, unpaired, Student's *t*-test. The trapezoidal rule was used to determine the area under the curve (AUC). For differences between plasma glucose and insulin concentrations during the glucose tolerance tests, a general linear model ANOVA was used for comparison and a Tukey's *post hoc t*-test to determine significance. Significance was determined as $P<0{\cdot}05$ for all analyses.

Results

Animal characteristics

PEPCK transgenic and PVG/c control rats were studied at 2 ages; young (4–5 weeks) and adult (12–14 weeks) to determine the effect of age on glucose metabolism. Young male PEPCK transgenic rats were significantly heavier than control rats (Table 1, $n=8$–13) that progressed to a greater increase as they reached adulthood. Adiposity levels (subcutaneous, infrarenal and gonadal fat pads) were not increased in young male transgenic rats but were significantly greater in the adult transgenics (Table 1, $n=8$–13). This increase in fat pad masses was associated with significantly higher circulating leptin concentrations that were not observed in younger transgenic rats (Table 1, $n=5$–7). Similar patterns were observed in the female rats (data not shown).

When energy balance components were measured, male PEPCK transgenic rats ate significantly more food than did the control rats at both the young (by 26%) and adult (by 23%) ages, but did not compensate with increases in total physical activity levels (Table 2). REE corrected for body weight (which makes up ∼70% of total energy expended) was not significantly different in young male PEPCK transgenic

Table 1 Body weight, white adipose tissue mass from subcutaneous, infrarenal and gonadal depots and circulating plasma leptin levels of young and adult male PVG/c control and phosphoenolpyruvate carboxykinase (PEPCK) transgenic rats

	Young		**Adult**	
	Control	PEPCK	Control	PEPCK
Body weight (g)	110·2 ± 6·6	123·0 ± 7·8[a]	319·4 ± 10·5	377·7 ± 4·6[a]
Subcutaneous (g)	0·44 ± 0·04	0·39 ± 0·04	2·19 ± 0·12	2·87 ± 0·13[a]
Infrarenal (g)	0·08 ± 0·01	0·09 ± 0·01	1·26 ± 0·09	2·49 ± 0·09[a]
Gonadal (g)	0·14 ± 0·03	0·13 ± 0·02	1·73 ± 0·08	2·79 ± 0·10[a]
Plasma leptin (ng/ml)	0·68 ± 0·08	0·79 ± 0·13	0·58 ± 0·21	2·68 ± 0·12[a]

Values are presented as mean ± S.E.M. ([a]$P<0{\cdot}05$ versus control rats ($n=5$–13)).

Table 2 Food intake, total physical activity, resting energy expenditure (REE) and substrate (fat and glucose) oxidation rates in young and adult male PVG/c control and PEPCK transgenic rats

	Young		Adult	
	Control	PEPCK	Control	PEPCK
Food intake (g/day)	13·6±0·6	17·2±0·7[a]	20·3±0·2	24·9±0·4[a]
Total activity (mvmts/day)	60 193±4095	69 721±3954	61 090±3048	59 965±2844
REE (kcal/day per kg)	454·1±14·8	523·7±26·6	280·6±3·7	253·7±7·8[a]
Fat oxidation (mg/min per kg)	9·3±2·3	14·5±3·4	7·1±0·5	9·0±1·7
Glucose oxidation (mg/min per kg)	49·5±7·4	49·3±6·4	25·5±0·7	16·7±2·5[a]

Values are presented as mean±S.E.M. ([a]$P<0·05$ versus control rats ($n=4–6$)).

compared with control rats, but was significantly lower in the adult PEPCK transgenic rats (Table 2). To determine the preferred fuel source during rest, substrate oxidation levels were measured. Young and adult PEPCK transgenic rats did not display any differences in Fox rates compared with the controls (Table 2). Gox rates were not different in the young PEPCK transgenic rats but were significantly lower as the rats aged (by 52%, $P<0·05$; Table 2). Similar patterns were observed in the female rats (data not shown).

Fasting plasma glucose and insulin concentrations were not different in young PEPCK transgenic compared with control rats (Table 3). As the rats aged, both fasting hyperglycaemia and hyperinsulinaemia were present in the PEPCK transgenic compared with control rats. Plasma triglyceride concentrations were also increased in the adult transgenic compared with control rats (0·18±0·03 vs 1·18±0·10 mmol/l, $n=4$, $P<0·05$).

Whole-body glucose turnover

Young PEPCK transgenic rats had increased basal EGP compared with age-matched PVG/c control rats (Fig. 1A). This was associated with an increase in glucose production from pyruvate, signifying an increased rate of total GNG (Fig. 1B). To determine if the main source of glucose was from the liver or the kidney, IPATTs were performed in young rats. Alanine is converted to glucose predominantly in the liver. Figure 1C shows that PEPCK and control rats had similar levels of glucose production from alanine, inferring that the increased glucose appearance in the basal state was attributable to elevated renal GNG.

Following a hyperinsulinaemic/euglycaemic clamp when both plasma glucose and insulin were matched (Table 3) both GIR (Fig. 1D) and EGP (Fig. 1E) were not significantly different between young PEPCK transgenic and age-matched PVG/c control rats. When the glucose infusion rate was calculated per steady-state insulin concentrations (GIR× clamped plasma insulin concentration), we found that there was no significant difference between the control and PEPCK transgenic rats (96·21±35·74 vs 80·93±40·23 μmol/min per kg×ng/ml, control versus PEPCK transgenic, $P>0·05$). Glucose uptake in fat (white and brown) was unaltered while there was a trend for an increase in muscle ($P=0·06$) in the PEPCK transgenic rats following insulin stimulation (Fig. 1F). Our results suggest that young PEPCK transgenic rats have increased basal glucose production, and when challenged with high insulin there was no difference with respect to glucose disposal or impact on glucose production compared with the control rats.

As the PEPCK transgenic rats aged, basal EGP remained significantly higher than control rats (Fig. 2A) despite significantly elevated plasma insulin concentrations (Table 3). When subjected to clamp conditions (Table 3), GIR (Fig. 2B) in the PEPCK transgenic rats was significantly reduced compared with the control rats. Whilst EGP was suppressed in the PEPCK rats from basal, it still remained significantly higher relative to the control rats under insulin-stimulated conditions due to the overexpression of a

Table 3 Circulating levels of glucose and insulin at basal and during the hyperinsulinaemic/euglycaemic clamp in young and adult PVG/c control and phosphoenolpyruvate carboxykinase (PEPCK) transgenic rats

	Young				Adult			
	Control		PEPCK		Control		PEPCK	
	Basal	Clamp	Basal	Clamp	Basal	Clamp	Basal	Clamp
Plasma glucose (mmol/l)	4·5±0·3	6·7±0·6[b]	4·5±0·3	7·2±0·7[b]	6·3±0·2	7·8±0·6[b]	7·2±0·4[a]	8·8±0·5[b]
Plasma insulin (ng/ml)	0·3±0·06	1·7±0·5[b]	0·2±0·05	1·7±0·6[b]	0·5±0·1	7·8±1·9[b]	1·5±0·4[a]	6·2±1·3[b]

Values are presented as mean±S.E.M. ([a]$P<0·05$ versus control rats under basal conditions; [b]$P<0·05$ versus corresponding basal levels ($n=4–8$)).

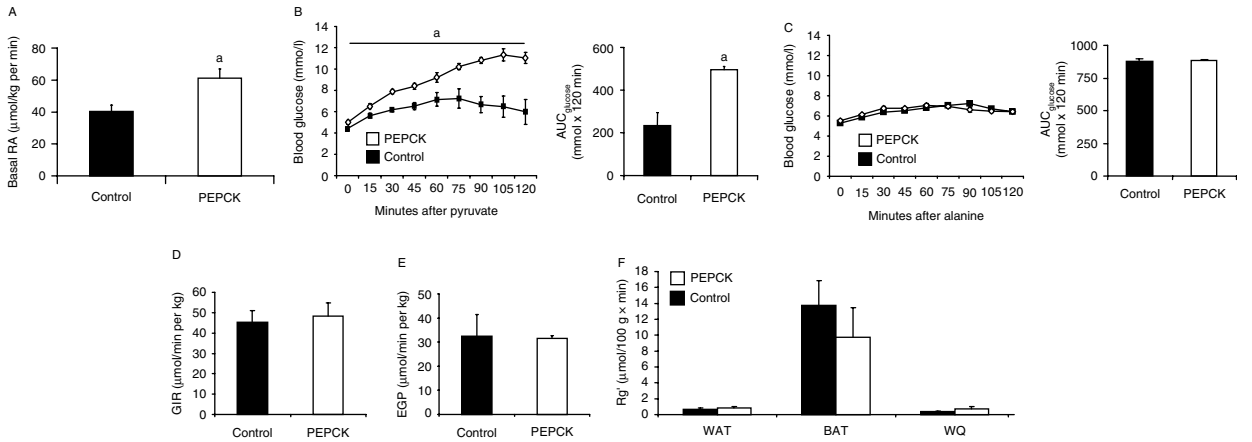

Figure 1 Whole-body glucose turnover under basal and insulin-stimulated conditions in young (4–5 weeks) PVG/c control and PEPCK transgenic rats. Basal rate of glucose appearance (R_a) (A), glucose production in response to a pyruvate tolerance test (inset total AUC_{glc}) (B) and glucose production in response to an alanine tolerance test (inset total AUC_{glc}) (C). Glucose infusion rate (GIR) (D), endogenous glucose production (EGP) levels (E) and glucose uptake into fat (white (WAT) and brown (BAT)) and muscle (WQ) (F) following a hyperinsulinaemic/euglycaemic clamp. PVG/c control rats ($n=5$–10) are shown in black and PEPCK transgenic rats ($n=4$–8) shown in white. Data presented as mean ± S.E.M. ($^aP<0{\cdot}05$ versus control).

non-suppressible PEPCK gene (Rosella *et al.* 1993; Fig. 2C). When the glucose infusion rate was calculated per steady-state insulin concentrations (GIR × clamped plasma insulin concentration), we found a significant reduction in the PEPCK transgenic rats compared with the PVG/c control rats, indicating insulin resistance and supporting our GIR and EGP data (607·24 ± 146·75 vs 257·22 ± 48·32 μmol/min per kg × ng/ml, control versus PEPCK transgenic, $P<0{\cdot}05$). Glucose uptake in muscle and both fat types was significantly lower in transgenic rats following insulin stimulation (Fig. 2D), implying peripheral insulin resistance and supporting our previous findings (Lamont *et al.* 2003). Adult PEPCK rats therefore, had increased basal glucose production, an impaired ability to suppress glucose production relative to the control rats and a decrease in glucose uptake in response to insulin stimulation.

To determine the contributing organ to the increased EGP in the adult rats, rates of alanine and glutamine conversion to glucose were measured basally and during the hyperinsulinaemic/euglycaemic clamps (Fig. 3A and B respectively). Under basal conditions there were no significant differences between the control and PEPCK transgenic rats in either alanine (liver; Fig. 3A) or glutamine (kidney; Fig. 3B) conversion to glucose (black bars with white dots), although there was a trend for the rate of alanine conversion to glucose ($\%R_{ala}$) to be higher in the PEPCK transgenic rats (Fig. 3A). Under clamp conditions (white bars with black dots), with matched insulin concentrations, $\%R_{ala}$ was significantly higher in the PEPCK transgenic rats. Similarly, compared with the control rats under clamp conditions, PEPCK transgenic rats were unable to suppress glutamine GNG. This suggests that in older PEPCK transgenic rats, the liver, and more so the kidney contributed significantly to an increase in EGP.

PEPCK, FBPase and G6Pase mRNA expression levels

We proceeded to investigate the mRNA levels of PEPCK, FBPase and G6Pase in both the liver and kidney of transgenic and control rats. As expected, Fig. 4A shows that young PEPCK transgenic rats had significantly elevated PEPCK mRNA in the liver and kidney compared with control rats. Interestingly, there was an approximate 1·7-fold increase in liver, and a fivefold increase in renal, expression of G6Pase mRNA in PEPCK rats ($P<0{\cdot}05$; Fig. 4B). Furthermore, FBPase mRNA levels were also up-regulated in both the liver (1·9-fold increase) and kidney (2·6-fold increase) of young PEPCK rats (Fig. 4C). When adult PEPCK rats were analysed, no significant difference in PEPCK, G6Pase or FBPase mRNA could be observed in the livers of these rats (Fig. 4D–F) that we suspect may be due to the increased plasma insulin at this age suppressing their expression. However, as expected, kidney PEPCK mRNA levels were found to be elevated in adult PEPCK transgenic rats (Fig. 4D), supporting our previous publications (Rosella *et al.* 1995, Lamont *et al.* 2003), with concomitant increases in both G6Pase (Fig. 4E) and FBPase mRNA expression levels (Fig. 4F).

Glucose-stimulated insulin secretion

An IVGTT was performed to examine the insulin secretory capacity of PEPCK transgenic rats (Fig. 5). Plasma glucose concentrations, as determined by total AUC ($AUC_{total\ glucose}$), were not significantly different in young rats (615·7 ± 23·7 vs 650·7 ± 16·4 mmol/l × 45 min, control versus PEPCK transgenic, $n=7$–15) but were significantly higher in adult transgenic compared with control rats (680·6 ± 12·5 vs 753·0 ± 21·3 mmol/l × 45 min, control versus PEPCK transgenic, $n=7$–15, $P<0{\cdot}05$). Insulin secretion in young rats was

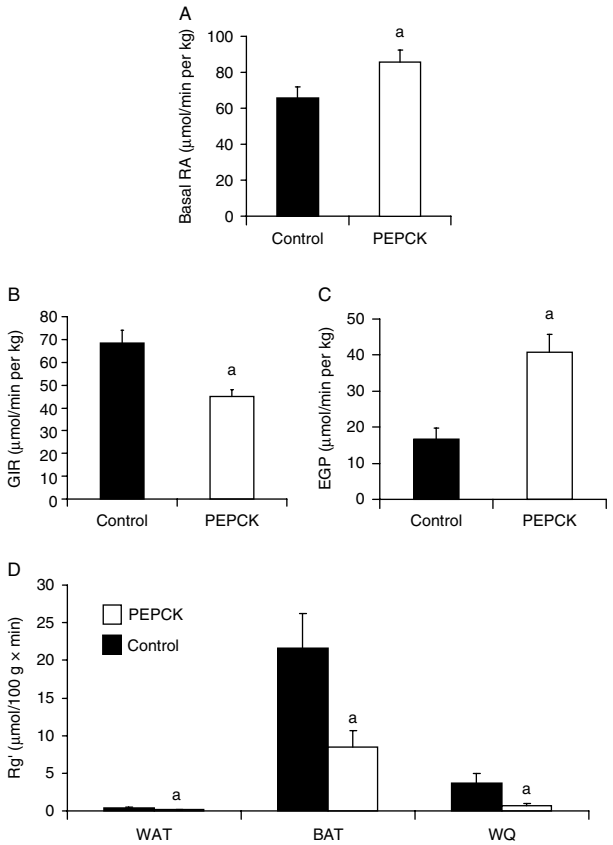

Figure 2 Whole-body glucose turnover under basal and insulin-stimulated conditions in adult (12–14 weeks) PVG/c control and PEPCK transgenic rats. Basal rate of glucose appearance (R_a) (A). Glucose infusion rate (GIR) (B), endogenous glucose production (EGP) levels (C) and glucose uptake into fat (white (WAT) and brown (BAT)) and muscle (WQ) (D) following a hyperinsulinaemic/euglycaemic clamp. PVG/c control rats ($n=5$–10) are shown in black and PEPCK transgenic rats ($n=4$–8) shown in white. Data presented as mean ± S.E.M. ($^aP<0{\cdot}05$ versus control).

not different (Fig. 5A). This was also reflected in similar AUCs of the first (0–5 min, AUC_{1st}) and second (5–40 min, AUC_{2nd}) phases between the two groups of rats (panel insets). As the rats aged, there was a significant defect in insulin secretion in the PEPCK transgenic rats compared with control rats (Fig. 5B). Interestingly, this defect was only detected in second-phase insulin secretion (panel inset), with first phase being normal.

Glucose tolerance

An IPGTT was performed at both ages to examine the glucose tolerance of PEPCK transgenic rats (Fig. 6). Young PEPCK transgenic rats displayed normal glucose tolerance (Fig. 6A). The corresponding plasma insulin concentrations were similarly unchanged (Fig. 6B). As the rats aged, there was a clear glucose intolerance in PEPCK transgenic compared with control rats, with a significant increase in total AUC_{glc} (Fig. 6C). This intolerance in transgenic rats occurred despite higher plasma insulin concentrations compared with control rats (Fig. 6D). Thus, the data clearly show that notwithstanding increased glucose production, young PEPCK transgenic rats had normal glucose tolerance. On the other hand, defects in insulin action and secretion were associated with glucose intolerance in older PEPCK transgenic rats.

Pair-feeding

To assess the effect of adiposity on glucose tolerance, we conducted a 12 week pair-feeding experiment from 4 weeks of age and the results presented in Table 4. Initial body weights were higher in PEPCK transgenic compared with control rats. Weight gain after 12 weeks of pair-feeding was comparable between control and pair-fed PEPCK transgenic rats and significantly lower compared with *ad libitum* fed PEPCK transgenic rats. Interestingly, at the end of the 12-week intervention period, both glucose intolerance and hyperinsulinaemia were present in both the pair-fed and *ad libitum* fed PEPCK transgenic groups compared with control rats (Table 4). This suggests that the glucose intolerance in the PEPCK transgenic rat is not entirely the result of the increased body weight.

Discussion

The primary defect in transgenic rats overexpressing PEPCK in the liver and kidneys is an increased rate of GNG, which contributes to an elevation in basal glucose production as early as 4 weeks of age. The elevation in EGP, which was measured by a combination of tracer kinetics and gluconeogenic substrate fluxes, is most likely attributable to increased GNG rather than glycogenolysis, given the overexpression of PEPCK transgenically and the prior overnight fast which

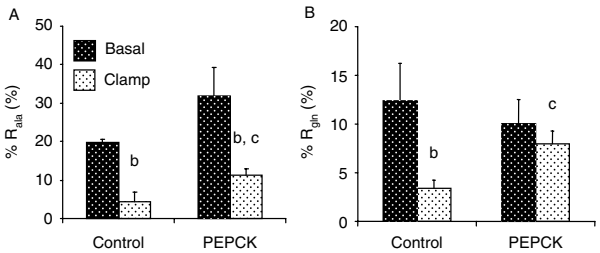

Figure 3 Determination of gluconeogenesis from liver and kidney in adult (12–14 weeks) PVG/c control and PEPCK transgenic rats. Rate of alanine conversion to glucose (%R_{ala}) (A) and rate of glutamine conversion to glucose (%R_{gln}) (B) in adult (12–14 weeks) PVG/c control ($n=4$–5) and PEPCK transgenic ($n=4$–5) rats under both basal conditions (black with white dots) and hyperinsulinaemic/euglycaemic clamp conditions (white with black dots). Data presented as mean ± S.E.M. ($^bP<0{\cdot}05$ versus basal turnover; $^cP<0{\cdot}05$ versus clamp PVG/c rats).

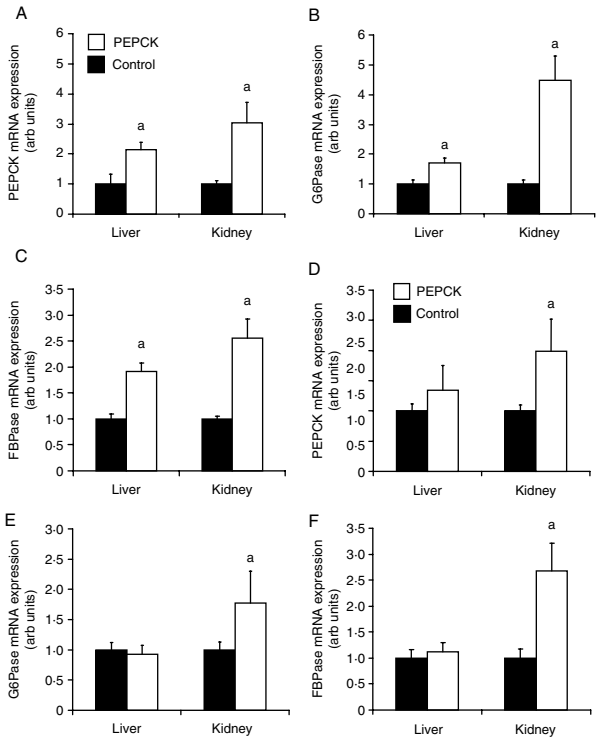

Figure 4 Total mRNA expression levels of the key gluconeogenic enzymes in young (A–C) and adult (D–F) PVG/c control rats and PEPCK transgenic rats. Total mRNA expression levels of PEPCK (A), G6Pase (B) and FBPase (C) in young PEPCK transgenic rats relative to young PVG/c rats in liver and kidney. Total mRNA expression levels of PEPCK (D), G6Pase (E) and FBPase (F) in adult PEPCK transgenic rats relative to adult PVG/c rats in liver and kidney. PVG/c control rats ($n=6$) are shown in black and PEPCK transgenic rats ($n=6$) shown in white. Data presented as mean ± S.E.M. ($^aP<0{\cdot}05$ versus control).

depletes hepatic glycogen. In fact pyruvate tolerance tests clearly showed that our young PEPCK transgenic rats maintained a higher plasma glucose concentration indicative of an increase in glucose production and supported the tracer kinetic studies. Further interrogation using alanine (which is specifically utilised by the liver) as a substrate, showed that young PEPCK transgenic rats did not have an increased glucose excursion implying that the liver was not the main organ that contributed to the overproduction of glucose. Unfortunately, we were unable to directly measure the contribution of the kidney using glutamine (which is the substrate of choice for the kidney), as this amino acid cannot be dissolved at a concentration that can be injected into animals. Thus, the only reasonable inference that can be made is that the kidney must be the main contributor to the overall glucose production in the young PEPCK transgenic rat.

Although increased abundance of PEPCK mRNA was to be expected, increased expression of FBPase and G6Pase were also noted in the liver and kidneys. This finding is similar to the reported data from Sun *et al.* (2002), who demonstrated that a comparable degree of PEPCK overexpression in the liver of mice to that of our transgenic rats (two- to three-fold), increased the expression of liver G6Pase. Sun proposed that PEPCK may be positively regulating the transcription of the other gluconeogenic enzymes, by impeding the insulin-mediated suppression of enzyme transcription (Sun *et al.* 2002). Positive associations between glucose production and gluconeogenic enzyme levels have also been suggested from other *in vivo* and *in vitro* models (Massillon *et al.* 1996, Argaud *et al.* 1997).

Despite elevated glucose appearance and the enzymatic machinery required to produce more glucose than controls, young PEPCK rats remained exquisitely sensitive to the suppressive effects of insulin on glucose production. The normal suppression of EGP in these young rats may be attributable to extrahepatic or 'indirect' effects of insulin, since much of the action of insulin to suppress hepatic glucose production is an indirect, systemic effect (Mittelman *et al.* 1997). Such processes include suppression of lipolysis,

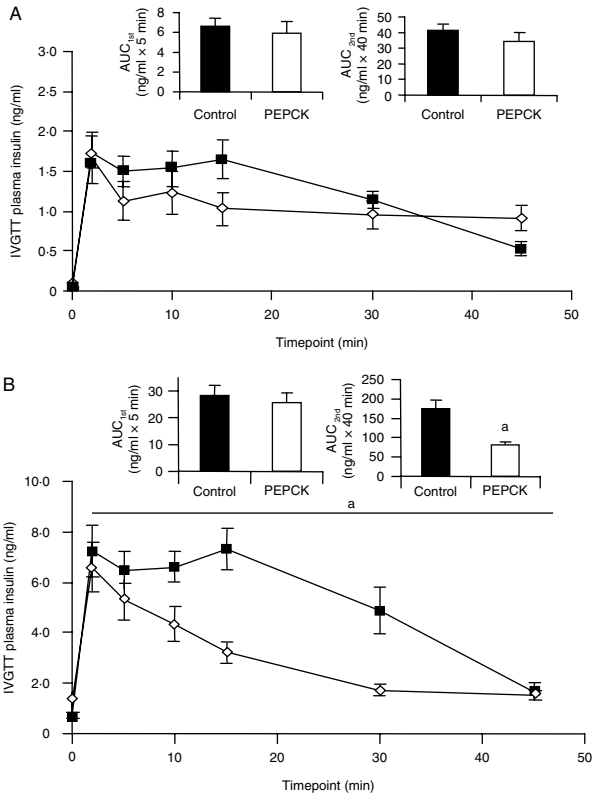

Figure 5 Glucose-mediated insulin secretion (IVGTT) in young and adult PVG/c control rats and PEPCK transgenic rats. Plasma insulin concentrations following an intravascular glucose tolerance test in young (A) and adult (B) PVG/c control and PEPCK transgenic rats. Area under curve for plasma insulin during first-phase (0–5 min; AUC_{1st}) and second-phase (5–40 min; AUC_{2nd}) illustrated as corresponding panel inserts. PVG/c control rats ($n=10$–11) are shown in black and PEPCK transgenic rats ($n=12$–15) shown in white. Data presented as mean ± S.E.M. ($^aP<0{\cdot}05$ versus controls).

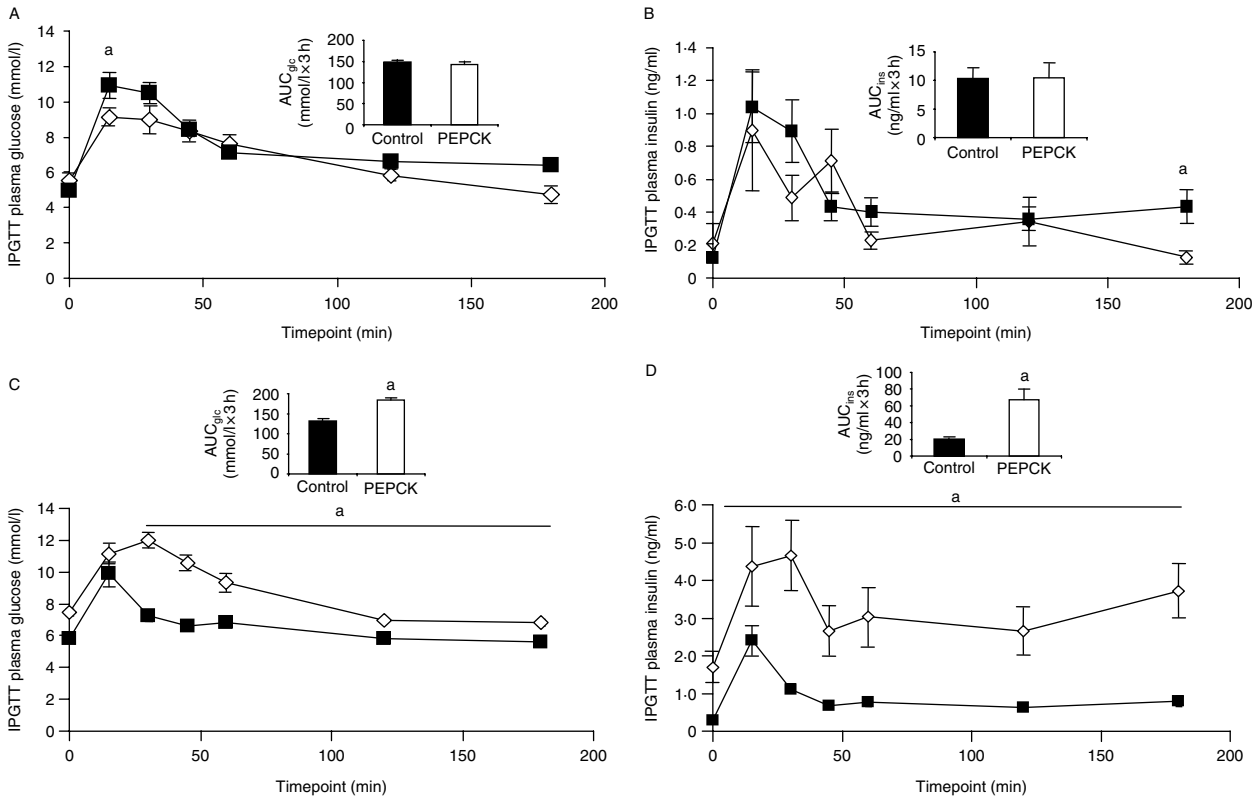

Figure 6 Glucose tolerance (IPGTT) in young and adult PVG/c control and PEPCK transgenic rats. Plasma glucose concentrations following an intraperitoneal glucose tolerance test in young (A) and adult (C) PVG/c control and PEPCK transgenic rats. Plasma insulin concentrations following an intraperitoneal glucose tolerance test in young (B) and adult (D) PVG/c control and PEPCK transgenic rats. Area under curve for plasma glucose (AUC_{glc}) and plasma insulin (AUC_{ins}) illustrated as corresponding panel inserts. Data for PVG/c control rats (n=10–15) are shown in black and for PEPCK transgenic rats (n=10–15) shown in white. Data presented as mean±S.E.M. ($^aP<0{\cdot}05$ versus controls).

inhibition of glucagon secretion and activation of hypothalamic descending pathways (Levine & Fritz 1956, Lewis *et al.* 1996, Mittelman *et al.* 1997, Obici *et al.* 2002, Buettner *et al.* 2005). It is important to highlight that clamp glucose concentrations were matched between the PEPCK transgenic and PVG/c control animals but were higher compared with the basal data (Table 3). Since it is known that glucose can also influence glucose utilisation through its mass action effect (Cherrington *et al.* 1978), this prevented us from making comparisons between basal and clamp conditions.

An important finding of the study is that enhanced glucose production was not associated with glucose intolerance in young PEPCK rats. Given that both peripheral glucose disposal and glucose-mediated insulin secretion from the pancreas (two major determinants of glucose tolerance) were preserved in the young rats the observation of normal glucose tolerance was, in hindsight, to be expected. It is not clear if the favourable adaptation to increased glucose production is partly driven by enhanced energy utilisation, as the trends towards increased physical activity and fuel oxidation at 4 weeks did

Table 4 Metabolic characteristics of PVG/c control and PEPCK transgenic rats that were either *ad libitum* fed or pair-fed to the control animals for 12 weeks starting from 4 weeks of age

	Control	PEPCK *ad libitum*	PEPCK pair-fed
Initial body weight (g)	71·7±1·1	90·4±1·1^a	83·6±1·1^a
12-Week weight gain (g)	275±34	328±10^a	288±5^b
Plasma glucose (mmol/l)	5·8±0·3	7·2±0·2^a	7·9±0·2^a
Plasma insulin (ng/ml)	1·0±0·2	2·6±0·3^a	2·2±0·2^a
$AUC_{glucose}$ (mmol/l×180 min)	1312±98	1683±41^a	1673±60^a
$AUC_{insulin}$ (ng/ml×180 min)	515±69	1270±78^a	749±87^{a,b}

Values are presented as mean±S.E.M. ($^aP<0{\cdot}05$ versus control rats; $^bP<0{\cdot}05$ versus PEPCK *ad libitum*-fed rats (n=5)).

not reach statistical significance. In addition, there was also a trend for increased glucose uptake in muscle, which together with the normal suppression of EGP contributed to the normal plasma glucose and insulin concentrations and glucose tolerance. Interestingly, the maintenance of normal glucose tolerance in the face of increased GNG is a phenomenon that our group also reported in hemizygous, liver-specific FBPase overexpressing mice (Lamont *et al.* 2006). Taken together, our results suggest that in rats genetically geared towards increased glucose production, glucose intolerance does not develop simply due to glucose excess. This implicates the ensuing defects of reduced glucose-mediated insulin secretion and/or peripheral insulin resistance as mandatory in the pathogenesis of glucose intolerance.

Consistent with this hypothesis, this study found that adult PEPCK rats only succumb to glucose intolerance with aging, which coincides with the development of increased body weight, insulin resistance and reduced glucose-mediated insulin secretion. The results from the pair-feeding studies suggest that the glucose intolerance is not significantly contributed to by obesity in the PEPCK transgenic rat. Rather, we suggest that it is more likely that excess glucose production causing glucose toxicity is the cause of glucose intolerance and insulin resistance in these transgenic rats. In accordance with our previous studies, the insulin resistance was found to affect muscle, fat and liver, resulting in both reduced glucose utilisation and impaired suppression of EGP (Lamont *et al.* 2003). Moreover, fat deposition in muscle (Lamont *et al.* 2003) was not elevated in the PEPCK transgenic rats implying that this reduced sensitivity to insulin cannot be attributable to ectopic fat accumulation. In contrast to our prior study of 14-week-old PEPCK rats, the current PEPCK rats exhibited elevated basal glucose production. In light of the unchanged gluconeogenic enzyme mRNA in the livers of older PEPCK rats, which may have been suppressed by the prevailing hyperinsulinaemia, the observed elevation in hepatic glucose production may have been facilitated by the increased availability of circulating triglyceride and/or triglyceride-derived substrates such as glycerol, which we previously documented in this model (Rosella *et al.* 1995). The development of mild hyperglycaemia, hyperinsulinaemia, hypertriglyceridaemia and a metabolic shift favouring increased whole-body lipid oxidation in this study are all consistent with the onset of insulin resistance in adult rats.

Impairment in first-phase glucose-mediated insulin secretion has long and repeatedly been recognised as an early sign of β-cell dysfunction contributing to the development of both glucose intolerance and diabetes

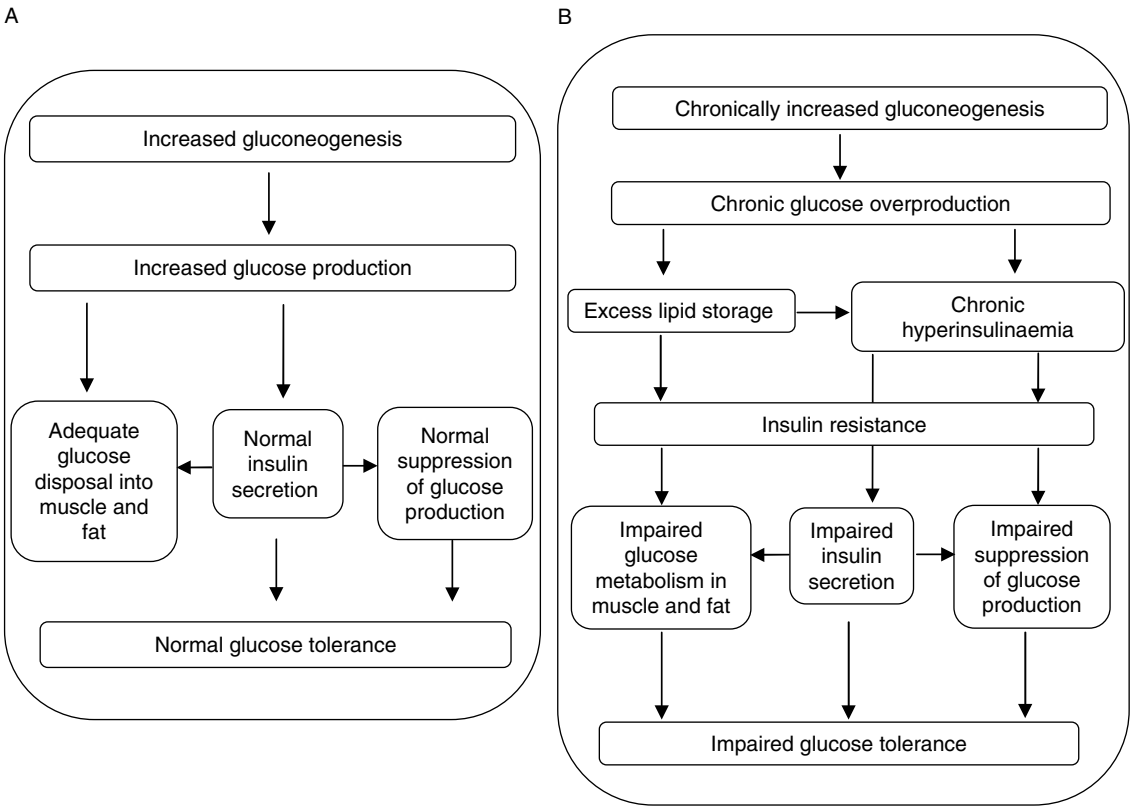

Figure 7 Summary of proposed sequence of events. Schematic diagram of the proposed theoretical sequence of events in young (A) and adult (B) PEPCK transgenic and PVG/c control rats.

(Calles-Escandon & Robbins 1987, Mitrakou *et al.* 1992, Gerich 1997, Bruttomesso *et al.* 1999). Second-phase glucose-mediated secretion is also severely suppressed in those with overt diabetes yet how it contributes to glucose intolerance has not been well established. An interesting and novel aspect of this study is that the impaired secretion that develops in PEPCK rats appears to be selective for second-phase insulin secretion with no effect on first-phase. Selective second-phase impairment in insulin secretion has previously been shown in mice fed a high-fat diet for 4 months (Wencel *et al.* 1995). Furthermore, Fontes *et al.* (2010) recently showed that old rats infused with a 70% glucose and intralipid infusion for 72 h also displayed a selection defect in second-phase insulin secretion associated with a reduction in insulin, *Pdx-1*, *Mafa*, *Glut2* and glucokinase gene expressions. Whilst our pair-feeding studies did not specifically assess glucose-mediated insulin secretion using an IVGTT, insulin secretion from the IPGTT was significantly reduced in the pair-fed PEPCK rats compared with the *ad libitum*-fed PEPCK rats implying an improvement in insulin secretory capacity. However, the pair-fed PEPCK rats still demonstrated increased insulin secretion compared with the PVG/c control rats suggesting that both obesity and glucose excess are potential contributors to the defective insulin secretion in states of impaired glucose tolerance. Whether the reduction in insulin concentration during the IPGTT in the pair-fed rats is due to improved second-phase secretion has not been determined in this study. Nonetheless, while the exact biochemical mechanism(s) underlying the reduction in second-phase insulin in the older PEPCK transgenic rat warrants further investigation, the presence of obesity and insulin resistance may be contributing factors.

Our results clearly show that early dysregulation of GNG has multiple secondary consequences, including the development of insulin resistance and defective insulin secretion, which act synergistically to worsen glucose tolerance. The sequence of events proposed that may explain the progression from enhanced glucose production to glucose intolerance (as illustrated in Fig. 7) is as follows: first, glucose overproduction in the young PEPCK transgenic rats is initially met with effective peripheral glucose utilisation and has no adverse consequences on insulin secretion or glucose tolerance. However, in the face of chronic hyperglycaemia, hyperinsulinaemia ensues, which promotes weight gain by stimulating the storage of ingested fatty acids as triglyceride in adipose tissue. The resulting obesity but more importantly excess glucose production (as suggested from our pair-feeding studies) may simultaneously compromise β-cell function and promote insulin resistance, resulting in reduced glucose utilisation. The elevated circulating triglycerides may increase the flux of gluconeogenic substrates to the liver, resulting in the acceleration of EGP due to elevated glucose output from both the liver and kidneys. Thus, chronic overproduction of glucose causing glucose toxicity-induced insulin resistance and impaired insulin secretion are factors which are ultimately responsible for the development of glucose intolerance in this model. Furthermore, our data support the recent clinical findings from Bock *et al.* (2007), who demonstrated that impaired insulin secretion and extrahepatic insulin resistance are the primary causes of postprandial hyperglycaemia in patients with increased GNG.

What does this mean for the treatment of diabetes? Patients with T2D who display significantly elevated fasting blood glucose have increased EGP (Golay *et al.* 1987). Our PEPCK transgenic rat model demonstrates that this defect not only contributes directly to hyperglycaemia, but also causes multiple secondary defects such as development of peripheral insulin resistance and a defect in insulin secretion that make diabetes difficult to control. Therefore, it follows that targeting elevated EGP early would be beneficial. Currently, metformin is the only medication whose primary target is suppression of GNG (Song *et al.* 2001, Proietto & Andrikopoulos 2004). Insulin suppresses EGP but causes weight gain and increases blood pressure (Randeree *et al.* 1992). Therefore, novel therapeutic agents that target glucose production are needed.

This study demonstrates that a primary increase in GNG caused by overexpression of a single gluconeogenic enzyme results in increased glucose production but does not lead to glucose intolerance. Insulin resistance and defects in insulin secretion must also be present for glucose intolerance to occur.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by a project grant from the National Health and Medical Research Council of Australia (NHMRC) and grants-in-aid from the Sir Edward Dunlop Medical Research Foundation and Diabetes Australia Research Trust (DART). S A was supported by a Career Development Award from the NHMRC.

Author contribution statement

S P M performed all body weight and food intake measurements, the intraperitoneal tolerance tests, basal glucose turnover studies, the mRNA expression analyses, acquired data and wrote the first draft; S H L performed the hyperinsulinaemic/euglycaemic clamps and together with C N J and S P M performed the intravascular tolerance tests and acquired data from these procedures; H M performed physical activity measurements and acquired data from this procedure; S N performed the gluconeogenesis clamp techniques and acquired data from these procedures; J P obtained funding and contributed to the study concept and design, provided critical revision of the manuscript for intellectual content; S A performed the surgeries, co-directed the study, contributed to the study concept and design, interpretation of the data and provided critical revision of the manuscript for intellectual content and B C F designed and co-directed the studies, performed the indirect calorimetry measurements, provided training and supervision for the animal physiology work, collated, interpreted and statistically analysed data, provided critical revision and intellectual input of the manuscript.

Acknowledgements

We thank Christian Rantzau, Amy Blair and Cassie Bush for excellent technical assistance.

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Received in final form 15 June 2011
Accepted 23 June 2011
Made available online as an Accepted Preprint
23 June 2011